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(54) Title: NUCLEIC ACID LIGANDS TO HEPATOCYTE GROWTH FACTOR/SCATTER FACTOR (HGF/SF) OR ITS RECEPTOR C-MET AND TO INTEGRINS

(57) Abstract: The invention provides nucleic acid ligands to hepatocyte growth factor/scatter factor (HGF) and its receptor c-met. The invention also provides nucleic acid ligands to integrins. The nucleic acid ligands of the instant invention are isolated using the SELEX method. SELEX is an acronym for Systematic Evolution of Ligands by EXponential enrichment. The nucleic acid ligands of the invention are useful as diagnostic and therapeutic agents.

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## NUCLEIC ACID LIGANDS TO HEPATOCYTE GROWTH FACTOR/SCATTER FACTOR (HGF/SF) OR ITS RECEPTOR C-MET AND TO INTEGRINS

### Field of the Invention

5 Described herein are methods for identifying and preparing high affinity nucleic acid ligands of hepatocyte growth factor/scatter factor (HGF) and its receptor c-met. The method utilized herein for identifying such nucleic acid ligands is called SELEX, an acronym for Systematic Evolution of Ligands by EXponential enrichment. The invention is also directed towards therapeutic and diagnostic reagents for diseases in which elevated  
10 HGF and c-met activity are causative factors.

Also described herein are methods for identifying nucleic acid ligands of integrins using the SELEX method. This aspect of the invention relates to integrin proteins, and methods and compositions for treating and diagnosing diseases involving integrins.

### Background of the Invention

Hepatocyte growth factor/scatter factor (abbreviated herein as HGF) is a potent cytokine which, through interaction with its receptor c-met, stimulates proliferation, morphogenesis, and migration of a wide variety of cell types, predominantly epithelial. HGF and c-met are involved in several cellular processes involved in tumorigenesis,  
20 notably angiogenesis and motogenesis, the latter having been implicated in the migration of cells required for metastasis (reviewed in Jiang and Hiscox (1997) *Histol Histopathol.* 12:537-55; Tamagnone and Comoglio (1997) *Cytokine Growth Factor Rev.* 8:129-42; Jiang, *et al.* (1999) *Crit Rev Oncol Hematol.* 29:209-48). Interestingly, proteases that degrade the extracellular matrix also activate HGF, which in turn up-regulates urokinase  
25 type plasminogen activator (uPA) and its receptor, resulting in an activating loop feeding the invasive and migratory processes required for metastatic cancer.

HGF and the c-met receptor are expressed at abnormally high levels in a large variety of solid tumors. In addition to numerous demonstrations *in vitro* of the effects of HGF/c-met on the behavior of tumor cell lines, the levels of HGF and/or c-met have been  
30 measured in human tumor tissues (reviewed in Jiang (1999) *Crit Rev Oncol Hematol.* 29:209-48). High levels of HGF and/or c-met have been observed in liver, breast,

pancreas, lung, kidney, bladder, ovary, brain, prostate, gallbladder and myeloma tumors in addition to many others.

For several of the cancer types listed above, the prognostic value of measuring HGF/c-met levels has been evaluated and found to be potentially useful for determining the progression and severity of disease. The correlative data are strongest in the case of breast cancer (Ghoussoub, Dillon *et al.* (1998) *Cancer*. 82:1513-20; Toi, Taniguchi *et al.* (1998) *Clin Cancer Res*. 4:659-64), and non-small cell lung cancer (Siegfried, Weissfeld *et al.* (1997) *Cancer Res*. 57:433-9; Siegfried, Weissfeld *et al.* (1998) *Ann Thorac Surg*. 66:1915-8).

Elevated levels of HGF and c-met have also been observed in non-oncological settings, such as hypertension (Morishita, Aoki *et al.* (1997) *J Atheroscler Thromb*. 4:12-9; Nakamura, Moriguchi *et al.* (1998), *Biochem Biophys Res Commun*. 242:238-43), arteriosclerosis (Nishimura, Ushiyama *et al.* (1997) *J Hypertens*. 15:1137-42; Morishita, Nakamura *et al.* (1998) *J Atheroscler Thromb*. 4:128-34), myocardial infarction (Sato, Yoshinouchi *et al.* (1998) *J Cardiol*. 32:77-82), and rheumatoid arthritis (Koch, Halloran *et al.* (1996) *Arthritis Rheum*. 39:1566-75), raising the possibility of additional therapeutic and diagnostic applications.

The role of HGF/c-met in metastasis has been elucidated in mice using cell lines transformed with HGF/c-met (reviewed in Jeffers, Rong *et al.* (1996) *J Mol Med*. 74:505-13). In another metastasis model, human breast carcinoma cells expressing HGF/c-met were injected in the mouse mammary fat pad, resulting in eventual lung metastases in addition to the primary tumor (Meiners, Brinkmann *et al.* (1998) *Oncogene*. 16:9-20). Also, transgenic mice which overexpress HGF become tumor-laden at many loci (Takayama, LaRochelle *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:701-6).

None of the data mentioned above provide proof of a direct causative role of HGF/c-met in human cancer, although the accumulated weight of the correlative data are convincing. However, a causal connection was established between germ-line c-met mutations, which constitutively activate its tyrosine kinase domain, and the occurrence of human papillary renal carcinoma (Schmidt, Duh *et al.* (1997) *Nat Genet*. 16:68-73).

Recent work on the relationship between inhibition of angiogenesis and the suppression or reversion of tumor progression shows great promise in the treatment of

cancer (Boehm, Folkman *et al.* (1997) *Nature*. 390:404-7). In this report, it was shown that the use of multiple angiogenesis inhibitors confers superior tumor suppression/regression compared to the effect of a single inhibitor. Angiogenesis is markedly stimulated by HGF, as well as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Rosen, Lamszus *et al.* (1997) *Ciba Found Symp.* 212:215-26). HGF and VEGF were recently reported to have an additive or synergistic effect on mitogenesis of human umbilical vein endothelial cells (HUVECs) (Van Belle, Witzembichler *et al.* (1998) *Circulation*. 97:381-90). Similar combined effects are likely to contribute to angiogenesis and metastasis.

Human HGF protein is expressed as a single peptide chain of 728 amino acids (reviewed in Mizuno and Nakamura (1993) *Exs.* 65:1-29; Rubin, Bottaro *et al.* (1993) *Biochim Biophys Acta*. 1155:357-71; Jiang (1999) *Crit Rev Oncol Hematol.* 29:209-48). The amino-terminal 31 residue signal sequence of HGF is cleaved upon export; followed by proteolytic cleavage by uPA and/or other proteases. The mature protein is a heterodimer consisting of a 463 residue  $\alpha$ -subunit and a 234 residue  $\beta$ -subunit, linked via a single disulfide bond. HGF is homologous to plasminogen: its  $\alpha$ -subunit contains an N-terminal plasminogen-activator-peptide (PAP) followed by four kringle domains, and the  $\beta$ -subunit is a serine protease-like domain, inactive because it lacks critical catalytic amino acids. The recently solved crystal structure of an HGF fragment containing PAP and the first kringle domain indicate that this region is responsible for heparin binding and dimerization (Chirgadze, Hepple *et al.* (1999) *Nat Struct Biol.* 6:72-9), in addition to receptor interaction.

Human c-met protein is exported to the cell surface via a 23 amino acid signal sequence (reviewed in Comoglio (1993) *Exs.* 65:131-65; Rubin (1993) *Biochim Biophys Acta*. 1155:357-71; Jiang (1999) *Crit Rev Oncol Hematol.* 29:209-48). The exported form of c-met is initially a pro-peptide which is proteolytically cleaved. The mature protein is a heterodimer consisting of an extracellular 50 kDa  $\alpha$ -subunit bound by disulfide bonds to a 140 kDa  $\beta$ -subunit. In addition to its extracellular domain, the  $\beta$ -subunit has a presumed membrane-spanning sequence and a 435 amino acid intracellular domain containing a typical tyrosine kinase.

HGF is produced primarily by mesenchymal cells, while c-met is mainly expressed on cells of epithelial origin. HGF is very highly conserved at the amino acid level between species. This homology extends into the functional realm as observed in mitogenic stimulation of hepatocytes in culture by HGF across species, including human, rat, mouse, pig and dog. This indicates that human HGF can be used cross-specifically in a variety of assays.

Given the roles of HGF and c-met in disease, it would be desirable to have agents that bind to and inhibit the activity of these proteins. It would also be desirable to have agents that can quantitate the levels of HGF and c-met in individual in order to gather diagnostic and prognostic information.

The integrins are a class of heterodimeric integral membrane proteins, one or more of which are expressed by most cell types (Hynes (1992) Cell 69:11-25). Some 16 homologous  $\alpha$  subunits and 8 homologous  $\beta$  subunits associate in various combinations to yield an extensive family of receptors. Each integrin heterodimer has a large extracellular domain that mediates binding to specific ligands. These ligands may include plasma proteins, proteins expressed on the surface of adjacent cells, or components of the extracellular matrix. Several of the integrins show affinity for more than one ligand and many have overlapping specificities (Hynes (1992) Cell 69:11-25). Both the  $\alpha$  and  $\beta$  subunits contribute to a small intracellular domain that contacts components of the actin cytoskeleton, thus forming a physical link between proteins outside and inside the cell.

Integrins play an important role in cellular adhesion and migration, and these properties are controlled by the cell, partly by modulation of integrin affinity for its ligands (so-called "inside-out" signaling). Conversely, the presence or absence of integrin ligation provides specific information about the cellular microenvironment, and in many instances integrins serve as a conduit for signal transduction. Ligand binding by an integrin may promote its incorporation into focal adhesions, the assembly of cytoskeletal and intracellular signaling molecules into supra-molecular complexes, and the initiation of a cascade of downstream signaling events including protein phosphorylation, calcium release, and an increase in intracellular pH (reviewed by Schwartz *et al.* (1995) Ann. Rev. Cell Dev. Biol. 11:549-99). Such "outside-in" signaling ties into pathways controlling cell proliferation, migration and apoptosis (Stromblad *et al.* (1996) J. Clin. Invest. 98:426-33;

Eliceiri *et al.* (1998) *J. Cell. Biol.* 140:1255-63). Integrins have been shown to play a role in such diverse physiological settings as embryonic development, wound healing, angiogenesis, clot formation, leukocyte extravasation, bone resorption and tumor metastasis.

5       The  $\beta_3$ -containing integrins are among the best studied of the receptor superfamily. The  $\beta_3$  subunit forms heterodimers with either  $\alpha_v$  ( $\alpha_v\beta_3$ ) or  $\alpha_{mb}$  ( $\alpha_{mb}\beta_3$ ). While these integrins show substantial overlap in ligand specificity, they play very different roles in normal physiology and in disease.

$\alpha_v\beta_3$  is expressed by activated endothelial cells, smooth muscle cells, osteoclasts,  
10   and, at a very low level, by platelets. It is also expressed by a variety of tumor cell types. The integrin binds to a number of plasma proteins or proteins of the extracellular matrix, many of which are associated with sites of inflammation or wound healing (Albelda (1991) *Am. J. Resp. Cell Mol. Biol.* 4:195-203). These include vitronectin, fibronectin, osteopontin, von Willebrand factor, thrombospondin, fibrinogen, and denatured collagen  
15   Type I (Hynes (1992) *Cell* 69:11-25). Each of these proteins share a common sequence motif, arginine-glycine-aspartic acid (RGD), that forms the core of the integrin binding site.

$\alpha_v\beta_3$  has been most intensely studied in the context of new blood vessel formation (angiogenesis) where it mediates the adhesion and migration of endothelial cells through  
20   the extracellular matrix. Angiogenesis in adults is normally associated with the cyclical development of the corpus luteum and endometrium and with the formation of granulation tissue during wound repair. In the latter case, microvascular endothelial cells form vascular sprouts that penetrate into the temporary matrix within a wound. These cells transiently express  $\alpha_v\beta_3$  and inhibition of the ligand binding function of the integrin  
25   temporarily inhibits the formation of granulation tissue (Clark *et al.* (1996) *Am. J. Pathol.* 148:1407-21). In cytokine-stimulated or unstimulated angiogenesis on the chick chorioallantoic membrane, blockade of  $\alpha_v\beta_3$  with a heterodimer-specific antibody prevents new vessel formation without affecting the pre-existing vasculature (Brooks *et al.* (1994) *Science* 264:569-71). Furthermore, the loss of adhesive contacts by endothelial cells  
30   activated for angiogenesis induces a phenotype characteristic of apoptotic cells (Brooks *et al.* (1994) *Cell* 79:1157-64); that is, ligand binding by  $\alpha_v\beta_3$  appears to transmit a survival

signal to the cell. Thus, adhesion and/or signaling mediated by  $\alpha_v\beta_3$  is essential for the formation of new blood vessels.

Solid tumors are unable to grow to significant size without an independent blood supply. It is currently hypothesized that the acquisition of an angiogenic phenotype is one of the limiting steps in the growth of primary tumors and of tumors at secondary sites (Folkman (1995) Nat. Med. 1:27-31). In addition, while the vasculature that penetrates a tumor mass provides a source of oxygen and nutrients, it also serves as a conduit for metastatic cells to leave the primary tumor and migrate throughout the body. Thus, inhibition of angiogenesis may limit both the growth and metastasis of cancerous lesions.

In experimental settings of tumor-induced angiogenesis, inhibition of ligand-binding by endothelial  $\alpha_v\beta_3$  prevented the formation of new blood vessels (Brooks *et al.* (1994) Cell 79:1157-64; Brooks *et al.* (1995) J. Clin. Invest. 96:1815-22), and inhibitors of  $\alpha_v\beta_3$  were shown to reduce the growth of experimental tumors *in vivo* (Brooks *et al.* (1995) J. Clin. Invest. 96:1815-22; Carron *et al.* (1998) Canc. Res. 58:1930-5).

$\alpha_v\beta_3$  is not only expressed by the microvasculature within tumors, but in some cases, is also found on the surface of tumor cells themselves. In particular, expression of  $\alpha_v\beta_3$  integrin has been detected in tissue sections from tumors of melanocytic and astroglial origin (Albelda *et al.* (1990) Canc. Res. 50:6757-64; Gladson and Cheresch (1991) J. Clin. Invest. 88:1924-32), and the level of integrin expression has been correlated with the stage or metastatic potential of the tumor (Albelda *et al.* (1990) Canc. Res. 50:6757-64; Gladson (1996) Am. J. Pathol. 148:1423-34; Hieken *et al.* (1996) J. Surg. Res. 63:169-73). Furthermore, melanoma cells grown *in vitro* in a three-dimensional matrix of denatured collagen undergo apoptosis upon  $\alpha_v\beta_3$  blockade.

Data such as these have driven an interest in inhibitors of  $\alpha_v\beta_3$  for the treatment of cancer. At present, two such inhibitors are in or near clinical trial: Vitaxin is a chimeric Fab fragment derived from the  $\alpha_v\beta_3$ -specific monoclonal antibody, LM609 (Wu *et al.* (1998) Proc. Nat. Acad. Sci. 95:6037-42). A phase I trial in late-stage cancer patients has been completed and no significant treatment-associated toxicities were observed (Gutheil *et al.* (1998) Am. Soc. Clin. Onc.). EMD121974 is a cyclic pentapeptide inhibitor of  $\alpha_v\beta_3$ . A Phase I study of this compound in Kaposi's sarcoma, brain tumors and solid tumors is scheduled to begin in 1999.

Angiogenesis (and  $\alpha_v\beta_3$ ) are implicated in the pathology of several other diseases, including psoriasis (Creamer *et al.* (1995) *Am. J. Pathol.* 147:1661-7), rheumatoid arthritis (Walsh *et al.* (1998) *Am. J. Pathol.* 152:691-702; Storgard *et al.* (1999) *J. Clin. Invest.* 103:47-54), endometriosis (Healy *et al.* (1998) *Hum. Reprod. Update* 4:736-40), and  
5 several proliferative diseases of the eye (Casaroli Marano *et al.* (1995) *Exp. Eye Res.* 60:5-17; Friedlander *et al.* (1996) *Proc. Nat. Acad. Sci.* 93:9764-9; Hammes *et al.* (1996) *Nat. Med.* 2:529-33). Inhibition of integrin ligand binding in each of these contexts may provide significant therapeutic benefit.

Atheromatous plaque and restenosis following angioplasty are pathologies  
10 characterized by thickening of the intima, the innermost layer of the arterial wall. The proliferation and/or migration of smooth muscle cells into the neointima with concomitant deposition of fibrous extracellular proteins contributes to vessel wall thickening and subsequent vessel occlusion. Platelets may also contribute to the development of restenotic lesions through adhesion to endothelial cells and the release of growth factors  
15 and cytokines that stimulate the underlying smooth muscle cell layer (Le Breton *et al.* (1996) *J. Am. Coll. Cardiol.* 28:1643-51).  $\alpha_v\beta_3$  integrin is expressed on arterial smooth muscle cells (Hoshiga *et al.* (1995) *Circ. Res.* 77:1129-35) and mediates their migration on vitronectin and osteopontin (Brown *et al.* (1994) *Cardiovasc. Res.* 28:1815-20; Jones *et al.* (1996) *Proc. Nat. Acad. Sci.* 93:2482-7; Liaw *et al.* (1995) *J. Clin. Invest.* 95:713-24;  
20 Panda *et al.* (1997) *Proc. Nat. Acad. Sci.* 94:9308-13), two matrix proteins associated with atherosclerotic tissues *in vivo* (Brown *et al.* (1994) *Cardiovasc. Res.* 28:1815-20; Giachelli *et al.* (1995) *Ann. N. Y. Acad. Sci.* 760:109-26; Panda *et al.* (1997) *Proc. Nat. Acad. Sci.* 94:9308-13). In addition,  $\alpha_v\beta_3$  expression on endothelial cells, and to a much lesser extent on platelets, is responsible for at least part of the adhesive interaction between  
25 these cell types (Le Breton *et al.* (1996) *J. Am. Coll. Cardiol.* 28:1643-51; Gawaz *et al.* (1997) *Circulation* 96:1809-18).  $\alpha_v\beta_3$  blockade with RGD-containing peptides or a monoclonal antibody was found to limit neointimal hyperplasia in several animal models of restenosis following arterial injury (Choi *et al.* (1994) *J. Vasc. Surg.* 19:125-34; Srivatsa *et al.* (1997) *Cardiovasc. Res.* 36:408-28; Slepian *et al.* (1998) *Circulation* 97:1818-27; Coleman *et al.* (1999) *Circ. Res.* 84:1268-76). Furthermore, treatment of  
30 patients undergoing percutaneous coronary intervention with an anti- $\beta_3$  antibody



(Reopro/abciximab/c7E3), which blocks both the platelet fibrinogen receptor,  $\alpha_{\text{IIb}}\beta_3$ , and  $\alpha_v\beta_3$ , provided long term reduction in the rates of death or myocardial infarction and in the rate of reocclusion of the artery (Lefkovits *et al.* (1996) *Am. J. Cardiol.* 77:1045-51), an effect that may be mediated through inhibition of  $\alpha_v\beta_3$  ligation. The observation that  $\alpha_v\beta_3$  is expressed by microvascular smooth muscle cells after experimentally-induced focal cerebral ischemia (Okada *et al.* (1996) *Am. J. Pathol.* 149:37-44) suggests that this integrin may also play some role in the development of ischemia/reperfusion injury in stroke.

Finally,  $\alpha_v\beta_3$  mediates the attachment of osteoclasts to matrix proteins, particularly osteopontin, on the surface of bone. Osteoclasts are responsible for the resorption of bone in normal physiology as well as in pathological conditions such as osteoporosis. A monoclonal antibody specific for  $\alpha_v\beta_3$  inhibited the binding and resorption of bone particles by osteoclasts *in vitro* (Ross *et al.* (1993) *J. Biol. Chem.* 268:9901-7). Furthermore, an RGD-containing protein, echistatin, was shown to block parathyroid-stimulated bone resorption in an animal model, as monitored by serum calcium levels (Fisher *et al.* (1993) *Endocrin.* 132:1411-3). Inhibitors of  $\alpha_v\beta_3$  integrin are thus considered of potential utility in treating debilitating bone loss such as occurs in osteoporosis.

$\alpha_{\text{IIb}}\beta_3$  (also referred to as GPIIb/IIIa) is the major integrin on the surface of platelets where it mediates the adhesion of activated platelets to the plasma protein fibrinogen (Nachman and Leung (1982) *J. Clin. Invest.* 69:263-9; Shattil *et al.* (1985) *J. Biol. Chem.* 260:11107-14). During clot formation, fibrinogen dimers cross-link platelets to one another through the integrin receptor.  $\alpha_{\text{IIb}}\beta_3$  also binds to several other plasma and cell matrix proteins, including von Willebrand factor, vitronectin, and fibronectin (Faull and Ginsberg (1996) *J. Am. Soc. Nephrol.* 7:1091-7).

Clot formation is a tightly regulated process that balances the need for rapid response to vascular injury, with the risk of aberrant occlusion of critical vessels. The  $\alpha_{\text{IIb}}\beta_3$  heterodimer is constitutively expressed on the surface of resting platelets at approximately 80,000 copies per cell (Wagner *et al.* (1996) *Blood* 88:907-14); however, the affinity of the integrin for fibrinogen is very low on these cells. Activation of platelets by ADP, epinephrine, collagen or thrombin leads to a dramatic enhancement in integrin ligand binding activity (Bennett and Vilaire (1979) *J. Clin. Invest.* 64:1393-401;

Integrilin and Aggrastat, have been approved for use in patients with acute coronary syndrome and/or in patients who are undergoing percutaneous coronary intervention. Reopro (Centocor/Eli Lilly) is a humanized murine monoclonal antibody Fab fragment with specificity for the  $\beta_3$  chain of  $\alpha_{IIb}\beta_3$ . Integrilin (COR Therapeutics) is a cyclic heptapeptide based on the integrin binding site of barbourin, an  $\alpha_{IIb}\beta_3$  inhibitory protein derived from snake venom. Aggrastat (Merck & Co.) is a non-peptide small molecule antagonist of the integrin. Unlike the small molecule inhibitors, Reopro cross-reacts with  $\alpha_v\beta_3$ , a fact that may account for the greater reduction in long-term rates of death and non-fatal myocardial infarction associated with its use (see above). A

significant effort is underway to identify new inhibitors of the platelet integrin with characteristics not found in the cohort of approved drugs. Specifically, compounds with specificity for the active, ligand-binding conformation of  $\alpha_{IIb}\beta_3$  may reduce the risk of bleeding complications associated with the existing anti-clotting therapies. Orally available compounds would be particularly useful for longer term therapy of patients at risk for recurrent myocardial infarction or unstable angina.

Given the role of integrins in the various disease states described above, it would be desirable to have high specificity inhibitors of particular integrins. The present invention provides such agents.

The dogma for many years was that nucleic acids had primarily an informational role. Through a method known as Systematic Evolution of Ligands by EXponential enrichment, termed the SELEX process, it has become clear that nucleic acids have three

dimensional structural diversity not unlike proteins. The SELEX process is a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules and is described in U.S. Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by EXponential Enrichment," now abandoned, U.S. Patent No. 5,475,096, entitled "Nucleic Acid Ligands," and U.S. Patent No. 5,270,163 (see also WO 91/19813), entitled "Methods for Identifying Nucleic Acid Ligands," each of which is specifically incorporated herein by reference in its entirety. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule.

The SELEX process provides a class of products which are referred to as nucleic acid ligands or aptamers, each having a unique sequence, and which has the property of binding specifically to a desired target compound or molecule. Each SELEX-identified nucleic acid ligand is a specific ligand of a given target compound or molecule. The SELEX process is based on the unique insight that nucleic acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can serve as targets. The SELEX method applied to the application of high affinity binding involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules, dissociating the nucleic acid-target complexes, amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific high affinity nucleic acid ligands to the target molecule.

Acid Ligands and Solution SELEX," describe a SELEX based method for selecting  
15 nucleic acid ligands containing photoreactive groups capable of binding and/or  
photocrosslinking to and/or photoinactivating a target molecule. U.S. Patent No.  
5,580,737, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between  
Theophylline and Caffeine," describes a method for identifying highly specific nucleic  
acid ligands able to discriminate between closely related molecules, which can be non-  
20 peptidic, termed Counter-SELEX. U.S. Patent No. 5,567,588, entitled "Systematic  
Evolution of Ligands by EXponential Enrichment: Solution SELEX," describes a  
SELEX-based method which achieves highly efficient partitioning between  
oligonucleotides having high and low affinity for a target molecule.

The SELEX method encompasses the identification of high-affinity nucleic acid  
25 ligands containing modified nucleotides conferring improved characteristics on the ligand,  
such as improved *in vivo* stability or improved delivery characteristics. Examples of such  
modifications include chemical substitutions at the ribose and/or phosphate and/or base  
positions. SELEX process-identified nucleic acid ligands containing modified nucleotides  
are described in U.S. Patent No. 5,660,985, entitled "High Affinity Nucleic Acid Ligands  
30 Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide  
derivatives chemically modified at the 5- and 2'-positions of pyrimidines. U.S. Patent No.

5,580,737, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH<sub>2</sub>), 2'-fluoro (2'-F), and/or 2'-methoxy (2'-OMe). U.S. Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2' Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in U.S. Patent No. 5,637,459, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chimeric SELEX," and U.S. Patent No. 5,683,867, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties of oligonucleotides with the desirable properties of other molecules.

The SELEX method further encompasses combining selected nucleic acid ligands with lipophilic compounds or non-immunogenic, high molecular weight compounds in a diagnostic or therapeutic complex as described in U.S. Patent No. 6,011,020, entitled "Nucleic Acid Ligand Complexes." Each of the above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated herein by reference in their entirety.

It is an object of the present invention to obtain nucleic acid ligands to HGF and c-met using the SELEX process.

It is a further object of the invention to obtain nucleic acid ligands that act as inhibitors of HGF and c-met.

It is a further object of the invention to provide therapeutic and diagnostic agents for tumorigenic conditions in which HGF and c-met are implicated.

It is yet a further object of the invention to use nucleic acid ligands to HGF and c-met to diagnose and treat hypertension, arteriosclerosis, myocardial infarction, and rheumatoid arthritis.

It is an even further object of the invention to use nucleic acid ligands to HGF singly or in combination with other nucleic acid ligands that inhibit VEGF and/or bFGF, and/or possibly other angiogenesis factors.

It is also an object of the present invention to provide methods that can be used to  
5 identify nucleic acid ligands that bind with high specificity and affinity to particular integrins.

It is a further object of the present invention to obtain nucleic acid ligands to particular integrins that inhibit the ability of that integrin to bind its cognate ligand.

It is a further object of the present invention to obtain integrin inhibiting  
10 pharmaceutical compositions for controlling thrombosis, tumor angiogenesis, tumor cell migration, proliferative ocular diseases, rheumatoid arthritis, psoriasis, osteoporosis, and restenosis.

It is yet a further object of the invention to obtain imaging agents for the non-invasive detection of deep vein or arterial thrombi.

15

#### **Summary of the Invention**

Methods are provided for generating nucleic acid ligands to HGF and c-met. The methods use the SELEX process for ligand generation. The nucleic acid ligands to HGF and c-met provided by the invention are useful as therapeutic and diagnostic agents for a  
20 number of diseases.

Methods are also provided for generating nucleic acid ligands to integrins, particularly to the  $\beta_3$  integrins. The methods use the SELEX process for ligand generation. Particular embodiments describe the isolation of nucleic acid ligand inhibitors of both  $\alpha_v\beta_3$  and  $\alpha_{IIb}\beta_3$ . The nucleic acid ligand inhibitors are derived from a library of 2'-fluoro-pyrimidine RNA sequences and were selected for high affinity binding to  $\alpha_v\beta_3$ . One of the  
25 modified nucleic acid ligands is shown to inhibit the binding of either vitronectin or fibrinogen to both of the purified integrins *in vitro*. This nucleic acid ligand binds to the surface of both resting and activated platelets with equivalent affinity and accumulates at the site of a preformed clot in an animal model of venous thrombosis.

The nucleic acid ligands to integrins provided by the invention are useful as therapeutic agents for a number of diseases including thrombosis and cancer. The nucleic acid ligands of the instant invention are also useful as diagnostic agents for thrombosis.

5

### **Brief Description of the Drawings**

Figure 1 illustrates the template and primer oligonucleotides used in the 2'-F-pyrimidine RNA SELEX experiments. The 5' fixed region of the template and primers contains a T7 promoter to facilitate transcription of RNA by T7 RNA polymerase.

Figure 2 illustrates RNaseH cleavage primers used in hybridization truncate SELEX. Bases depicted in bold-type are 2'-Ome modified and bases underlined are deoxyribonucleosides. The random region is designated as "N". Upon treatment with RNaseH, the fixed regions are removed at the positions indicated by the carets. Note that there are two possible cleavage sites at the 5'-end of the fixed region, resulting in RNA which has one or two fixed G residues.

Figure 3 illustrates binding of SELEX pools to HGF. Figure 3A shows HGF SELEX 1 30N7 pools and Figure 3B shows HGF SELEX 2 30N8 pools.

Figure 4 illustrates two methods of evaluating HGF SELEX 3 30N7 pool binding to HGF. In Figure 4A, heparin competes with RNA pools for binding to 2.7 nM HGF and Figure 4B illustrates conventional pool binding.

Figure 5 illustrates two methods of evaluating HGF SELEX 3 30N7 pool binding to HGF. Figure 5A shows that tRNA competes with RNA pools for binding to 2.7 nM HGF and Figure 5B shows conventional pool binding.

Figure 6 illustrates inhibition of 10 ng/mL HGF stimulation of starved HUVECs by aptamers. Figure 6A shows a 1<sup>st</sup> set of aptamers and Figure 6B illustrates a 2<sup>nd</sup> set of aptamers.

Figure 7 illustrates truncates of aptamer 8-102 (SEQ ID NO:12). Figure 7A shows predicted two-dimensional structures of full-length and truncated sequences and Figure 7B shows binding of full-length and truncated aptamers to HGF.

Figure 8 illustrates truncates of aptamer 8-17 (SEQ ID NO:68). Figure 8A shows a predicted two-dimensional structures of full-length and truncated sequences and Figure 8B shows binding of full-length and truncated aptamers to HGF.

Figure 9 illustrates binding of HGF truncate SELEX pools. Figure 9A shows the HGF SELEX 4 30N7 series and Figure 9B shows the HGF SELEX 5 30N7 series.

Figure 10 shows aptamer inhibition of 100 ng/mL HGF stimulation of 4MBr-5 cells.

Figure 11 illustrates aptamer inhibition of 50 ng/mL HGF stimulation of 4MBr5  
5 cells. Figure 11A shows the effect of PEGylation of 36mer and Figure 11B shows a comparison of PEGylated 36mer to best full-length inhibitor 8-17.

Figure 12 shows aptamer inhibition of 50 ng/mL HGF stimulation of 4MBr-5 cells.

Figure 13 shows HUVEC mitogenesis by 10 ng/mL HGF, 10 ng/mL VEGF, or both HGF and VEGF.

10 Figure 14 illustrates aptamer-mediated inhibition of HUVEC mitogenesis. Figure 14A shows stimulation by both HGF and VEGF inhibited by either HGF or VEGF aptamers or both. Figure 14B illustrates stimulation by HGF alone inhibited by either HGF or VEGF aptamer or both. Figure 14C illustrates stimulation by VEGF alone inhibited by either HGF or VEGF aptamer or both.

15 Figure 15 depicts ratios of selected to unselected partially 2'-OMe substituted purines in aptamer NX22354.

Figure 16 illustrates 2'-OMe substituted derivatives of NX22354 binding to HGF (average of two experiments).

Figure 17 illustrates binding of SELEX pools to c-met. Figure 17A shows c-Met  
20 SELEX 40N7; Figure 17B shows c-Met SELEX 30N8; and Figure 17C shows both SELEXes: a, c pools, 40N7; b, d pools, 30N8.

Figure 18 illustrates binding of c-met SELEX pools to c-met and KDR Ig fusion proteins.

Figure 19 shows binding of c-met 40N7 cloned aptamers to c-met and KDR Ig  
25 fusion proteins. Figure 19A shows clone 7c-1 and Figure 19B shows clone 7c-3.

Figure 20 illustrates the binding of affinity-enriched RNA pools to immobilized  $\alpha_v\beta_3$ . 5'-biotinylated RNA pools were incubated at varying concentrations in 96-well microtiter plates coated with integrin  $\alpha_v\beta_3$ . Bound RNAs were detected via the biotin moiety by a chromogenic assay. Data are expressed in absorbance units at 405 nm as a  
30 function of input RNA concentration.



Figure 21 illustrates cross-reactivity of aptamer 17.16 (SEQ ID NO:249) to purified integrin  $\alpha_{\text{mb}}\beta_3$ . 5'-biotinylated aptamer 17.16 was incubated at varying concentrations in microtiter wells coated with either integrin  $\alpha_v\beta_3$  or  $\alpha_{\text{mb}}\beta_3$ . Bound RNA was detected via the biotin moiety using a chromogenic assay. Data are expressed as the per cent of the maximum signal to normalize for differences in protein coating.

Figure 22 illustrates cross-reactivity of aptamer 17.16 (SEQ ID NO:249) to purified integrin  $\alpha_v\beta_3$ . 5'-biotinylated aptamer 17.16 or a control RNA of similar length and base composition were incubated at varying concentrations in microtiter wells coated with either  $\alpha_v\beta_3$  or  $\alpha_{\text{mb}}\beta_3$ . Bound RNAs were detected via the biotin moiety by a chromogenic assay. Data are expressed in absorbance units at 405 nm as a function of input RNA concentration.

Figure 23 illustrates  $\beta_3$  aptamer inhibition of integrin ligand binding. Biotinylated fibrinogen or vitronectin were incubated in microtiter wells coated with either integrin  $\alpha_v\beta_3$  or  $\alpha_{\text{mb}}\beta_3$  in the presence or absence of varying concentrations of ligand binding competitors. Competitors included aptamer 17.16 (SEQ ID NO:249), a control RNA of similar length and base composition, a cyclic RGD peptide (cRGD, see Materials and Methods), an  $\alpha_v\beta_3$ -specific monoclonal antibody (LM609), or unmodified fibrinogen or vitronectin. Bound ligands were detected via biotin using a chromogenic assay. Data are expressed in absorbance units at 405 nm as a function of input competitor concentration.

Figure 23A illustrates competition of vitronectin binding to immobilized  $\alpha_v\beta_3$ ; Figure 23B illustrates competition of fibrinogen binding to immobilized  $\alpha_v\beta_3$ ; and Figure 23C illustrates competition of fibrinogen binding to immobilized  $\alpha_{\text{mb}}\beta_3$ . An estimate of the maximum absorbance value was determined for each ligand/integrin pair in the absence of competitor. The baseline absorbance value was determined by adding 5 mM EDTA to the incubation buffer. The maximum and minimum values so determined were (a) 0.914/0.113; (b) 1.042/0.122; and (c) 0.889/0.128.

Figure 24 illustrates binding of aptamer 17.16 (SEQ ID NO:249) to activated or resting human platelets. 5'-fluorescein-conjugated aptamer 17.16 or a control RNA of similar length and base composition were incubated at various concentrations with resting or thrombin-activated human platelets ( $10^6/\text{mL}$ ). Incubations were at room temperature in buffered saline containing divalent cations, 0.1% BSA and 0.01% sodium azide. Mean

the concentration of aptamer or control RNA.

5        Figure 25 illustrates biodistribution of [ $^{99m}\text{Tc}$ ]-aptamer 17.16 (SEQ ID NO:249) or control RNA in a rabbit venous clot model. A clot derived from human platelet-rich plasma was generated *in situ* by temporary isolation of the jugular vein of an anesthetized rabbit. After restoration of circulation over the clot, [ $^{99m}\text{Tc}$ ]-labeled aptamer or control RNA were injected into the bloodstream of the rabbit via the ipsilateral ear vein. After one  
10    hour, the animal was sacrificed and tissues were weighed and counted in a gamma counter. Accumulation of radioactivity in various tissues is reported as the percentage of the injected dose per gram wet weight of tissue.

### **Detailed Description of the Preferred Embodiments**

15        The central method utilized herein for identifying nucleic acid ligands is called the SELEX process, an acronym for Systematic Evolution of Ligands by Exponential enrichment. As applied to identifying ligands to HGF and c-met the method comprises: a) contacting the candidate mixture of nucleic acids with HGF or c-met, or expressed domains or peptides corresponding to HGF or c-met; b) partitioning between members of  
20    said candidate mixture on the basis of affinity to HGF or c-met; and c) amplifying the selected molecules to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity for binding to HGF or c-met. As applied to identifying integrins the method comprises: a) contacting the candidate mixture of nucleic acids with integrins, or expressed domains or peptides corresponding to integrins; b) partitioning  
25    between members of said candidate mixture on the basis of affinity to integrins; and c) amplifying the selected molecules to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity for binding to integrins.

Definitions

Various terms are used herein to refer to aspects of the present invention. To aid in the clarification of the description of the components of this invention, the following definitions are provided.

5       As used herein, "**nucleic acid ligand**" is a non-naturally occurring nucleic acid having a desirable action on a target. Nucleic acid ligands are often referred to as "**aptamers**". The term aptamer is used interchangeably with nucleic acid ligand throughout this application. A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way which  
10       modifies/alters the target or the functional activity of the target, covalently attaching to the target as in a suicide inhibitor, facilitating the reaction between the target and another molecule. In the preferred embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which  
15       predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule. In the present invention, the targets are HGF, c-met and integrins or portions thereof. Nucleic acid ligands include nucleic acids that are identified from a candidate mixture of nucleic acids, said nucleic acid ligand being a ligand of a  
20       given target, by the method comprising: a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and c) amplifying the increased affinity nucleic acids to yield a ligand-enriched  
25       mixture of nucleic acids.

As used herein, "**candidate mixture**" is a mixture of nucleic acids of differing sequence from which to select a desired ligand. The source of a candidate mixture can be from naturally-occurring nucleic acids or fragments thereof, chemically synthesized nucleic acids, enzymatically synthesized nucleic acids or nucleic acids made by a  
30       combination of the foregoing techniques. In a preferred embodiment, each nucleic acid

has fixed sequences surrounding a randomized region to facilitate the amplification process.

As used herein, "**nucleic acid**" means either DNA, RNA, single-stranded or double-stranded, and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"**SELEX**" methodology involves the combination of selection of nucleic acid ligands that interact with a target in a desirable manner, for example binding to a protein, with amplification of those selected nucleic acids. Optional iterative cycling of the selection/amplification steps allows selection of one or a small number of nucleic acids which interact most strongly with the target from a pool which contains a very large number of nucleic acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. In the present invention, the SELEX methodology is employed to obtain nucleic acid ligands to HGF, c-met and integrins or portions thereof. The SELEX methodology is described in the SELEX Patent Applications.

"**SELEX target**" or "**target**" means any compound or molecule of interest for which a ligand is desired. A target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, etc. without limitation. In this application, the SELEX targets are HGF, c-met and integrins. In particular, the SELEX targets in this application include purified integrins, HGF and c-met, and fragments thereof, and short peptides or expressed protein domains comprising HGF or c-met. Also includes as targets are fusion proteins comprising portions of HGF or c-met and other proteins.

As used herein, "s **lid support**" is defined as any surface to which molecules may be attached through either covalent or non-covalent bonds. This includes, but is not limited to, membranes, microtiter plates, magnetic beads, charged paper, nylon, Langmuir-Bodgett films, functionalized glass, germanium, silicon, PTFE, polystyrene, gallium arsenide, gold, and silver. Any other material known in the art that is capable of having functional groups such as amino, carboxyl, thiol or hydroxyl incorporated on its surface, is also contemplated. This includes surfaces with any topology, including, but not limited to, spherical surfaces and grooved surfaces.

As used herein, "**HGF**" refers to hepatocyte growth factor/scatter factor. This includes purified hepatocyte growth factor/scatter factor, fragments of hepatocyte growth factor/scatter factor, chemically synthesized fragments of hepatocyte growth factor/scatter factor, derivatives or mutated versions of hepatocyte growth factor/scatter factor, and fusion proteins comprising hepatocyte growth factor/scatter factor and another protein. "HGF" as used herein also includes hepatocyte growth factor/scatter factor isolated from species other than humans.

As used herein "**c-met**" refers to the receptor for HGF. This includes purified receptor, fragments of receptor, chemically synthesized fragments of receptor, derivatives or mutated versions of receptor, and fusion proteins comprising the receptor and another protein. "c-met" as used herein also includes the HGF receptor isolated from a species other than humans.

As used herein "**integrin**" refers to any integrin. This includes purified integrins, fragments of integrins, chemically synthesized fragments of integrins, derivatives or mutated versions of integrins, and fusion proteins comprising the integrin and another protein. "Integrins" as used herein also includes integrins isolated from a species other than humans.

Note that throughout this application, various references are cited. Every reference cited herein is specifically incorporated in its entirety.

#### SELEX

In the preferred embodiment, the nucleic acid ligands of the present invention are derived from the SELEX methodology. The SELEX process is described in U.S. Patent

Application Serial No. 07/536,428, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, U.S. Patent No. 5,475,096, entitled "Nucleic Acid Ligands" and U.S. Patent No. 5,270,163 (see also WO 91/19813), entitled "Methods for Identifying Nucleic Acid Ligands." These applications, each specifically incorporated  
5 herein by reference, are collectively called the SELEX Patent Applications.

The SELEX process provides a class of products which are nucleic acid molecules, each having a unique sequence, and each of which has the property of binding specifically to a desired target compound or molecule. Target molecules are preferably proteins, but can also include among others carbohydrates, peptidoglycans and a variety of small  
10 molecules. SELEX methodology can also be used to target biological structures, such as cell surfaces or viruses, through specific interaction with a molecule that is an integral part of that biological structure.

In its most basic form, the SELEX process may be defined by the following series of steps:

- 15 1) A candidate mixture of nucleic acids of differing sequence is prepared. The candidate mixture generally includes regions of fixed sequences (i.e., each of the members of the candidate mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are chosen either: a) to assist in the amplification steps described below; b) to mimic a sequence known to bind to the target;  
20 or c) to enhance the concentration of a given structural arrangement of the nucleic acids in the candidate mixture. The randomized sequences can be totally randomized (i.e., the probability of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).
- 25 2) The candidate mixture is contacted with the selected target under conditions favorable for binding between the target and members of the candidate mixture. Under these circumstances, the interaction between the target and the nucleic acids of the candidate mixture can be considered as forming nucleic acid-target pairs between the target and those nucleic acids having the strongest affinity for the target.
- 30 3) The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with lesser affinity to the target. Because only an extremely small

number of sequences (and possibly only one molecule of nucleic acid) corresponding to the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the nucleic acids in the candidate mixture (approximately 5-50%) are retained during partitioning.

- 5           4) Those nucleic acids selected during partitioning as having the relatively higher affinity for the target are then amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively higher affinity for the target.

- 5) By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer unique sequences, and the average degree of  
10   affinity of the nucleic acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a small number of unique nucleic acids representing those nucleic acids from the original candidate mixture having the highest affinity to the target molecule.

- The basic SELEX method has been modified to achieve a number of specific  
15   objectives. For example, U.S. Patent Application Serial No. 07/960,093, filed October 14, 1992, now abandoned, and U.S. Patent No. 5,707,796, both entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describe the use of the SELEX process in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. U.S. Patent Application Serial No.  
20   08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," now abandoned, U.S. Patent No. 5,763,177 and U.S. Patent No. 6,001,577, both entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX" all describe a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or  
25   photocrosslinking to and/or photoinactivating a target molecule. U.S. Patent No. 5,580,737, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, termed Counter-SELEX. U.S. Patent No. 5,567,588, entitled "Systematic Evolution of Ligands by  
30   Exponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low

ligands containing modified nucleotides concerning improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified nucleic acid ligands containing modified nucleotides are described in U.S. Patent No. 5,660,985, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. U.S. Patent No. 5,637,459, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH<sub>2</sub>), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). U.S. Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2' Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in U.S. Patent No. 5,637,459, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," and U.S. Patent No. 5,683,867, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

In U.S. Patent No. 5,496,938, methods are described for obtaining improved nucleic acid ligands after the SELEX process has been performed. This patent, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," is specifically incorporated herein by reference in its entirety.



One potential problem encountered in the diagnostic use of nucleic acids is that oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes such as endonucleases and exonucleases before the desired effect is manifest. Certain chemical modifications of the nucleic acid ligand can be made to increase the *in vivo* stability of the nucleic acid ligand or to enhance or to mediate the delivery of the nucleic acid ligand. See, e.g., U.S. Patent Application Serial No. 08/117,991, filed September 8 1993, now abandoned and U.S. Patent No. 5,660,985, both entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," and the U.S. Patent Application Serial No. 09/362,578, filed July 28, 1999, entitled "Transcription-free SELEX," each of which is specifically incorporated herein by reference. Modifications of the nucleic acid ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping. In preferred embodiments of the instant invention, the nucleic acid ligands are RNA molecules that are 2'-fluoro (2'-F) modified on the sugar moiety of pyrimidine residues.

The modifications can be pre- or post-SELEX process modifications. Pre-SELEX process modifications yield nucleic acid ligands with both specificity for their SELEX target and improved *in vivo* stability. Post-SELEX process modifications made to 2'-OH nucleic acid ligands can result in improved *in vivo* stability without adversely affecting the binding capacity of the nucleic acid ligand. Other modifications are known to one of ordinary skill in the art. Such modifications may be made post-SELEX process (modification of previously identified unmodified ligands) or by incorporation into the SELEX process.

In some embodiments, the nucleic acid ligands become covalently attached to their targets upon irradiation of the nucleic acid ligand with light having a selected wavelength. Methods for obtaining such nucleic acid ligands are detailed in U.S. Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," now abandoned, U.S. Patent No. 5,763,177 and U.S. Patent No. 6,001,577, both  
5 entitled "Systematic Evolution of Nucleic Acid Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX," each of which is specifically incorporated herein by reference in its entirety.

The nucleic acid ligands of the invention are prepared through the SELEX  
10 methodology that is outlined above and thoroughly enabled in the SELEX applications incorporated herein by reference in their entirety.

#### Nucleic acid ligands to HGF and c-met

The SELEX process can be performed using purified HGF or c-met, or fragments  
15 thereof as a target. Alternatively, full-length HGF or c-met, or discrete domains of HGF or c-met, can be produced in a suitable expression system. Alternatively, the SELEX process can be performed using as a target a synthetic peptide that includes sequences found in HGF or c-met. Determination of the precise number of amino acids needed for the optimal nucleic acid ligand is routine experimentation for skilled artisans.

20 In preferred embodiments, the SELEX process is carried out using HGF or c-met attached to a solid support. A candidate mixture of single stranded RNA molecules is then contacted with the solid support. In especially preferred embodiments, the single stranded RNA molecules have a 2'-fluoro modification on C and U residues, rather than a 2'-OH group. After incubation for a predetermined time at a selected temperature, the solid  
25 support is washed to remove unbound candidate nucleic acid ligand. The nucleic acid ligands that bind to the HGF or c-met protein are then released into solution, then reverse transcribed by reverse transcriptase and amplified using the Polymerase Chain Reaction. The amplified candidate mixture is then used to begin the next round of the SELEX process.

30 In the above embodiments, the solid support can be a nitrocellulose filter. Nucleic acids in the candidate mixture that do not interact with the immobilized HGF or c-met can

be removed from this nitrocellulose filter by application of a vacuum. In other embodiments, the HGF or c-met target is adsorbed on a dry nitrocellulose filter, and nucleic acids in the candidate mixture that do not bind to the HGF or c-met are removed by washing in buffer. In other embodiments, the solid support is a microtiter plate  
5 comprised of, for example, polystyrene.

In still other embodiments, the HGF or c-met protein is used as a target for Truncate SELEX, described in U.S. Patent Application Serial No. 09/275,850, filed March 24 1999, entitled "Truncation SELEX Method," incorporated herein by reference in its entirety.

10 In preferred embodiments, the nucleic acid ligands thus obtained are assayed for their ability to inhibit the HGF/c-met interaction. In one embodiment, this is accomplished by performing a cell migration assay. Certain cell types, such as A549 lung carcinoma cells, will show increased migration through a Matrigel-coated filter insert (Becton Dickinson) in the presence of HGF. Thus, the degree of inhibition of HGF  
15 activity in the presence of an HGF or c-met nucleic acid ligand can be assayed by determining the number of cells that have migrated through the filter in the presence of HGF.

20 Methods and compositions for using nucleic acid ligands to HGF and c-met to treat and diagnose disease

Given that elevated levels of c-met and HGF are observed in hypertension, arteriosclerosis, myocardial infarction, and rheumatoid arthritis, nucleic acid ligands will serve as useful therapeutic and diagnostic agents for these diseases. In some  
25 embodiments, inhibitory nucleic acid ligands of HGF and c-met are administered, along with a pharmaceutically accepted excipient to an individual suffering from one of these diseases. Modifications of these nucleic acid ligands are made in some embodiments to impart increased stability upon the nucleic acid ligands in the presence of bodily fluids. Such modifications are described and enabled in the SELEX applications cited above.

In other embodiments, nucleic acid ligands to HGF and c-met are used to measure  
30 the levels of these proteins in an individual in order to obtain prognostic and diagnostic information. Elevated levels of c-met and HGF are associated with tumors in the liver,

breast, pancreas, lung, kidney, bladder, ovary, brain, prostate, and gallbladder. Elevated levels of HGF and c-met are also associated with myeloma.

In other embodiments, nucleic acid ligands that inhibit the HGF/c-met interaction are used to inhibit tumorigenesis, by inhibiting, for example, angiogenesis and  
5 motogenesis.

In one embodiment of the instant invention, a nucleic acid ligand to HGF is used in combination with nucleic acid ligands to VEGF (vascular endothelial growth factor) and/or bFGF (basic fibroblast growth factor) to inhibit tumor metastasis and angiogenesis. The use of multiple nucleic acid ligands is likely to have an additive or synergistic effect  
10 on tumor suppression. Nucleic acid ligands that inhibit VEGF are described in U.S. Patent No. 5,849, 479, U.S. Patent No. 5,811,533 and U.S. Patent Application Serial No. 09/156,824, filed September 18, 1998, each of which is entitled "High Affinity Oligonucleotide Ligands to Vascular Endothelial Growth Factor," and each of which is specifically incorporated herein by reference in its entirety. Nucleic acid ligands to VEGF  
15 are also described in U.S. Patent No. 5,859,228, U.S. Patent No. 6,051,698, U.S. Patent Application Serial No. 08/870,930, filed June 6, 1997 and U.S. Patent Application Serial No. 09/254,968, filed March 16 1999, each of which is entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes, and each of which is specifically incorporated by reference in its entirety. Nucleic acid ligands to bFGF are described in  
20 U.S. Patent No. 5,639,868 and U.S. Patent Application Serial No. 08/442,423, filed May 16, 1995, both entitled "High Affinity RNA ligands for Basic Fibroblast Growth Factor," each of which is specifically incorporated herein by reference in its entirety.

#### Nucleic acid ligands to integrins

25 The SELEX process can be performed using purified integrins, or fragments thereof as a target. Alternatively, full-length integrins, or discrete domains of integrins, can be produced in a suitable expression system. Alternatively, the SELEX process can be performed using as a target a synthetic peptide that includes sequences found in an integrin. Determination of the precise number of amino acids needed for the optimal  
30 nucleic acid ligand is routine experimentation for skilled artisans.

In preferred embodiments, the SELEX process is carried out using integrins attached to polystyrene beads. A candidate mixture of single stranded RNA molecules is then contacted with the beads. In especially preferred embodiments, the single stranded RNA molecules have a 2'-fluoro modification on C and U residues, rather than a 2'-OH group.

5 After incubation for a predetermined time at a selected temperature, the beads are washed to remove unbound candidate nucleic acid ligands. The nucleic acid ligands that bind to the integrin are then released into solution, reverse transcribed by reverse transcriptase and amplified using the Polymerase Chain Reaction (PCR). The amplified candidate mixture is then used to begin the next round of the SELEX process. Example 5 illustrates one  
10 possible way of performing the SELEX process using integrins as targets.

In preferred embodiments, the nucleic acid ligands thus obtained are assayed for their ability to inhibit the interaction of the integrin with its cognate ligand. In one embodiment, this is accomplished by first coating microtiter plates with the appropriate integrin(s). A ligand for the integrin, such as vitronectin or fibrinogen, is then biotinylated  
15 and contacted with the coated integrin in the presence of the nucleic acid ligand to be assayed. After incubation for a suitable period of time, the microtiter plate is washed, and the amount of vitronectin or fibrinogen binding to integrin is quantitated by adding a streptavidin-alkaline phosphatase conjugate, followed by a colorimetric substrate for alkaline phosphatase, such as p-nitrophenyl phosphate. The alkaline phosphatase signal in  
20 each well of the plate is thus inversely proportional to the effectiveness of the nucleic acid ligand as an inhibitor of the interaction between the bound integrin and its cognate ligand.

In other embodiments, the nucleic acid ligands are analyzed using binding to human platelets as an assay. This can be done, for example, by fluorescently labeling the nucleic acid ligand by any of the numerous techniques known in the art. The fluorescent nucleic  
25 acid ligand is then contacted with platelets, and the amount of nucleic acid ligand is be quantitated using Fluorescence Activated Cell Sorting (FACS).

The distribution of the nucleic acid ligands of the instant invention can also be studied *in vivo*. In some embodiments, nucleic acid ligands are labeled with a radiolabel used in the art of radioimaging. For example, a nucleic acid ligand can be conjugated to  
30 the isotope <sup>99m</sup>Tc using one of a number of techniques known in the art. The radiolabelled nucleic acid can then be studied in an animal model of venous thrombosis. For example, a

human blood clot can be generated in rabbit vein by first isolating the vein *in situ* by ligation, and then infusing the vein with human platelet-rich plasma and heparin to induce the formation of a blood clot. Blood flow through the vein is then re-established, and the radiolabelled nucleic acid ligand is introduced into the animals blood supply. The  
5 distribution of the radiolabelled nucleic acid ligand can then be studied in the rabbit's tissues to determine whether the nucleic acid ligand has accumulated in the clot, rather than in other areas.

10 Methods and compositions for using nucleic acid ligands to integrins to treat and diagnose disease

The nucleic acid ligands provided by the instant invention have a number of potential uses as therapeutic and diagnostic agents. In some embodiments, nucleic acid ligands that inhibit the interaction between platelet-expressed integrins and their cognate ligands are administered, along with pharmaceutically accepted excipients, in order to  
15 prevent the formation of blood clots in patients susceptible to deep vein thrombosis. In other embodiments, the nucleic acid ligands are used to treat acute thrombosis formation during and following percutaneous coronary intervention. In still other embodiments, the nucleic acid ligands of the invention are used to treat patients with acute coronary syndromes such as unstable angina or myocardial infarction.

20 In other embodiments, radiolabelled nucleic acid ligands to platelet-expressed integrins are administered to individuals who are to undergo major surgery, or have suffered major trauma. Such nucleic acid ligands can function as imaging agents for the detection of thrombi, by showing sites in the body where large aggregations of platelets are present. If a thrombosis is detected by radioimaging at a critical site in the body, then  
25 anticoagulant and thrombolytic treatment --including treatment with the inhibitory nucleic acid ligands of the instant invention-- can be given locally. The advantage of using such a nucleic acid ligand imaging agent is that the anticoagulant and thrombolytic treatments --which can cause harm if administered prophylactically by allowing internal bleeding to continue without efficient clotting-- can be given only to those individuals who definitely  
30 have a dangerous thrombosis. Moreover, these treatments can be specifically injected at the site where the thrombosis has been detected by the nucleic acid ligand, instead of

injecting higher concentrations into the bloodstream in the hope that some active agent will be carried to all potential sites of thrombosis.

Nucleic acid ligands to  $\alpha_v\beta_3$  integrin can be used to inhibit tumor growth and metastasis. They can also be used to treat ocular diseases including, but not limited to, diabetic retinopathy, retinopathy of prematurity, and macular degeneration. Other diseases for which  $\alpha_v\beta_3$  nucleic acid ligands are useful therapeutic agents include, but are not limited to, endometriosis, psoriasis, rheumatoid arthritis, stroke, osteoporosis, and restenosis.

The following examples are given by way of illustration only. They are not to be taken as limiting the scope of the invention in any way.

### Examples

#### Example 1. Generation of nucleic acid ligands to HGF and c-met

##### Materials and Methods

In the sections below entitled "Results: HGF" and "Results: c-met", the following materials and methods were used.

Proteins. The HGF protein and c-met-IgG<sub>1</sub>-His<sub>6</sub> fusion protein, which were used in the SELEX process, and the KDR-IgG<sub>1</sub>-His<sub>6</sub> proteins were purchased from R&D Systems, Inc. (Minneapolis, MN). The human c-met-IgG<sub>1</sub>-His<sub>6</sub> fusion protein --described from the amino to the carboxyl terminus-- consists of 932 amino acids from the extracellular domains of the  $\alpha$  and  $\beta$  chains of c-met, a factor Xa cleavage site, 231 amino acids from human IgG<sub>1</sub> (Fc domain), and a (His)<sub>6</sub> tag. This protein is referred to in the text and figures as c-met. A similar fusion protein containing the vascular endothelial growth factor receptor KDR will be referred to as KDR.

Anti-HGF monoclonal antibody MAB294 was purchased from R&D Systems, Inc. Human IgG<sub>1</sub> was produced in-house by stable expression from Chinese hamster ovary cells.

SELEX templates and primers. Standard SELEX templates carrying 30 or 40 random nucleotides flanked by fixed regions of the N7 or N8 series and associated primers (Figure 1) were used as described (Fitzwater and Polisky (1996) Methods Enzymol.

267:275-301). Truncate SELEX was done by the hybridization method described in U.S. Patent Application Serial No. 09/275,850, filed March 24 1999, entitled "Truncation SELEX Method," incorporated herein by reference in its entirety, using RNaseH cleavage primers (Figure 2).

5        SELEX methods. Initial HGF SELEX experiments were done by two closely-related partitioning methods, both involving separating free from bound RNA on nitrocellulose filters. Conventional SELEX involves mixing target protein and RNA library in HBSMC buffer (hepes-buffered saline, 25 mM hepes, 137 mM NaCl, 5 mM KCl plus 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.4), followed by filtration on nitrocellulose under  
10 vacuum. Maintaining vacuum, the filter is washed in buffer, followed by vacuum release and RNA extraction. In spot filter SELEX, the protein is applied to a dry nitrocellulose 13 mm filter, allowed to adsorb for several minutes, then pre-incubated in Buffer S (HBSMC buffer plus 0.02% each of ficoll, polyvinylpyrrolidone, and human serum albumin) for 10 minutes at 37°C to remove unbound protein. The wash buffer is removed, and then the  
15 RNA library is added in the same buffer, and incubated with the protein-bound filter. The filters are washed by repeated incubations in fresh buffer, followed by RNA extraction.

SELEX was initiated with between 1 and 5 nmoles of 2'-fluoro-pyrimidine RNA sequence libraries containing either a 30 or 40 nucleotide randomized region sequence (Figure 1). The RNA libraries were transcribed from the corresponding synthetic DNA  
20 templates that were generated by Klenow extension (Sambrook, Fritsch *et al.* (1989) 3:B.12). The DNA templates were transcribed in 1 mL reactions, each containing 0.25 nM template, 0.58 μM T7 RNA polymerase, 1 mM each of ATP and GTP, 3 mM each of 2'-F-CTP and 2'-F-UTP, 40 mM Tris-HCl (pH 8.0), 12 mM MgCl<sub>2</sub>, 1 mM spermidine, 5 mM DTT, 0.002% Triton X-100 and 4% polyethylene glycol (w/v) for at least 4 hours at 37°C.  
25 The full-length transcription products were purified by denaturing polyacrylamide gel electrophoresis. Radiolabeled RNA was obtained from transcription reactions as described above, but containing 0.2 nM ATP and 100 μCi of α-<sup>32</sup>P-ATP. Alternatively, radiolabeled RNA was obtained by labeling the 5'-end of RNA with α-<sup>32</sup>P -ATP (NEN-DuPont), catalyzed by T4 polynucleotide kinase (New England Biolabs). To prepare RNA  
30 containing 5'-OH groups for kinase reactions, transcription reactions included 5 mM guanosine.



For conventional filter SELEX, radiolabeled RNA pools were suspended in HBSMC buffer to which HGF protein was added, and incubated at 37°C for 30 minutes to 3 hours depending on the round. Binding reactions were then filtered under suction through 0.45 µm nitrocellulose filters (Millipore), pre-wet with binding buffer. The filters were immediately washed with at least 5 mL of HBSMC buffer. For each binding reaction, a protein-minus control reaction was done in parallel in order to determine the amount of background binding to the filters. The amount of RNA retained on the filters was quantified by Cherenkov counting, and compared with the amount input into the reactions. Filter-retained RNA was extracted with phenol and chloroform, and isolated by ethanol precipitation in the presence of 1-2 µg glycogen.

The isolated RNA was subsequently used as a template for avian myeloblastosis virus reverse transcriptase (AMV-RT, Life Sciences) to obtain cDNA. One hundred pmoles of the 3'-primer (Figure 1) was added to the RNA and annealed by heating for 3 minutes at 70°C, followed by chilling on ice. The 50 µL reaction contained 5 U AMV-RT, 0.4 mM each of dNTPs, 50 mM Tris-HCl (pH 8.3), 60 mM NaCl, 6 mM Mg(OAc)<sub>2</sub>, and 10 mM DTT, which was incubated for 45 minutes at 48°C. The cDNA was amplified by PCR with the 5'- and the 3'-primers (Figure 1), and the resulting DNA template was transcribed to obtain RNA for the next round of SELEX.

To minimize selection of undesirable nitrocellulose-binding sequences, beginning in round three, pools were pre-soaked with nitrocellulose filters before incubating with the target protein. This treatment worked well to control background binding and helped ensure that each SELEX round had a positive signal/noise ratio. The progress of SELEX was monitored by nitrocellulose filter-binding analysis of the enriched pools (see below).

Truncate SELEX was performed by the hybridization method described in U.S. Patent Application Serial No. 09/275,850, filed March 24 1999, entitled "Truncation SELEX Method," incorporated herein by reference in its entirety. Briefly, 2'-F-RNA pools were body-labeled during transcription and cleaved by RNaseH using specific cleavage primers to remove the fixed sequences from the SELEX pool (Figure 2). This RNA was then bound to target protein HGF and recovered following partitioning as in a conventional filter SELEX experiment. The recovered RNA was then biotinylated at its 3' end and hybridized overnight under appropriate conditions with single-stranded full-length

complementary strand DNA obtained from the starting SELEX pool, from which the RNA had been transcribed. The RNA/DNA complexes were then captured on streptavidin-coated magnetic beads and extensively washed to remove non-hybridized DNA. The bound DNA in the captured RNA/DNA complexes was then eluted by heat denaturation and amplified using conventional SELEX PCR primers. To complete the cycle, the resulting DNA was then used as a transcription template for generating RNA to be cleaved by RNaseH, and used in the next round of truncate SELEX.

For plate SELEX, a polystyrene well was pre-blocked in 400  $\mu$ L of blocking agent for 60 minutes at 37°C. The blocking agent was removed and the desired amount of RNA in 100  $\mu$ L binding buffer was added and incubated for 60 minutes at 37°C. White, polystyrene breakaway wells (catalog #950-2965) used for partitioning were from VWR (Denver, CO). The blocking agents, I-block and Superblock, were purchased from Tropix (Bedford, MA) and Pierce (Rockford, IL), respectively. The preadsorption was done to remove any nucleic acids which might bind to the well or the blocking agent. The random and round one libraries were not preadsorbed to plates to avoid loss of unique sequences. C-met protein was diluted in HBSMCK (50 mM HEPES, pH 7.4, 140 mM NaCl, 3 mM KCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ ), and was adsorbed to polystyrene wells by incubating 100  $\mu$ L of diluted protein per well for 60 minutes at 37°C. The wells were each washed with three 400  $\mu$ L aliquots of HIT buffer (HBSMCK, 0.1% I-block, 0.05 % Tween 20), and then blocked in 400  $\mu$ L of blocking agent for 60 minutes at 37°C. SELEX was initiated by incubating 100  $\mu$ L of RNA in the protein-bound well for 60 minutes at 37°C. The RNA was removed and the wells were washed with 400  $\mu$ L aliquots of HIT buffer. Increasing numbers of washes were used in later rounds. The wells were then washed twice with 400  $\mu$ L water. RNA bound to c-met was eluted by adding 100  $\mu$ L water and heating at 95°C for 5 minutes and then cooled on ice, followed by reverse transcription.

Nitrocellulose filter-binding. In binding reactions, RNA concentrations were kept as low as possible --between 1 and 20 pM-- to ensure equilibrium in conditions of protein excess. Oligonucleotides were incubated for 15 minutes at 37°C with varying amounts of the protein in 43  $\mu$ L of the binding buffer. Thirty-two microliters of each binding mixture was placed on pre-wet 0.45  $\mu$ m nitrocellulose filters under suction. Each well was immediately washed with 0.5 mL binding buffer. The amount of radioactivity retained on

the filters was quantitated by imaging. The radioactivity that bound to filters in the absence of protein was used for background correction. The percentage of input oligonucleotide retained on each filter spot was plotted against the corresponding log protein concentration. The nonlinear least square method was used to obtain the dissociation constant ( $K_d$ , reference Jellinek, Lynott *et al.* (1993) Proc. Natl. Acad. Sci. USA. 90:11227-31).

Competitor titration curves were generated essentially as a standard binding curve, except that the protein and RNA concentrations were kept constant, and the competitor concentration was varied. Competitors were also added at a fixed concentration in binding experiments to increase stringency for purposes of comparing pool binding affinities. In these experiments, the competitor concentration was chosen based on the results from the competitor titration curves.

Molecular cloning and DNA sequencing. To obtain individual sequences from the enriched pools, the PCR products from the final SELEX rounds were cloned using one of two blunt-end cloning kits: Perfectly Blunt (Novagen, Madison, WI), or PCR-Script (Stratagene, La Jolla, CA). Clones were sequenced with the ABI Prism Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequences were obtained from an automated ABI sequencer, and text files were collated and analyzed by computer alignment and inspection.

Boundary determinations. 5' and 3' boundaries of RNA aptamers were determined by the method of partial alkaline hydrolysis as described (Jellinek, Green *et al.* (1994) Biochemistry. 33:10450-6).

Cell assays. Standard cell culture procedures were employed in the course of performing *in vitro* experiments to test aptamer-mediated inhibition of HGF activity. For cell migration assays, monolayers of A549 (lung carcinoma) cells were grown on the top-sides of Matrigel-coated filter inserts (Becton Dickinson, Franklin Lakes, NJ) in 24-well plates. The cells adhere to the upper surface of the filter, which is placed in growth medium containing HGF. After two days, the cells are physically removed from the top surface of the filter. The filter is then removed from the insert and stained with crystal violet. Since all cells on the top of the filter are gone, the only cells that remain are those

that are have migrated to the bottom of the filter. In the presence of HGF, significantly more cells are found on the bottom of the filter compared to controls without HGF.

Oligonucleotide synthesis and modification RNA was routinely synthesized by standard cyanoethyl chemistry as modified (Green, Jellinek *et al.* (1995) Chem Biol. 2:683-95). 2'-Fluoro-pyrimidine phosphoramidite monomers were obtained from JBL Scientific (San Luis Obispo, CA); 2'-OMe purine, 2'-OH purine, hexyl amine, and the dT polystyrene solid support were obtained from Glen Research (Sterling, VA).

For addition of 40K(40,000)-PEG, RNA oligomers were synthesized with an amino-linker at the 5'-position. This was subsequently reacted with NHS-ester 40K-PEG manufactured by Shearwater Polymers, Inc. (Huntsville, AL), and purified by HPLC on a reverse-phase preparative column.

2'-O-methyl purine substitution Determination of which 2'-OH-purines can be substituted by 2'-OMe purine was done as described (Green (1995) Chem Biol. 2:683-95). Briefly, a set of oligonucleotides was synthesized with a mixture of 2'-OMe amidites and 2'-OH amidites at defined purine positions. The set was designed so that each oligonucleotide contains a subset of partially-substituted purines, and the complete set encompasses all purines. Each aptamer was 5'-end labeled and subjected to limited alkaline hydrolysis followed by binding to HGF protein at two different concentrations, 50 and 100 pM. Following binding, protein-bound RNA was separated by standard nitrocellulose filtration. Bound RNA was recovered and analyzed by high-resolution gel electrophoresis. The fragmented alkaline-hydrolyzed aptamers which were not exposed to HGF were run to establish the cleavage patterns of the unselected aptamers. Hydrolysis occurs only at 2'-OH-purines. If a given position requires 2'-OH for optimal binding to HGF, it appears as a relatively darker band compared to the unselected aptamer at that position.

## Example 2. Results – HGF

Five HGF SELEX experiments were done in total. The first three were done by conventional filter SELEX, while the latter two were done by the hybridization truncate SELEX method described in U.S. Patent Application Serial No. 09/275,850, filed March 24 1999, entitled "Truncation SELEX Method," incorporated herein by reference in its

entirety. HGF SELEX 1 was done with 30N7 2'-F-RNA for thirteen rounds of conventional filter binding. HGF SELEX 2 was done with 30N8 2'-F-RNA for thirteen rounds of conventional filter binding. HGF SELEX 3 was done with 30N7 2'-F-RNA for seven rounds by spot filter binding, followed by eight rounds of filter binding. HGF SELEX 4 was done by hybridization filter SELEX for three rounds, starting with pool 8 from HGF SELEX 1. HGF SELEX 5 was done by hybridization filter SELEX for three rounds, starting with pool 11 from HGF SELEX 3. HBSMC buffer was used in conventional SELEX reactions, and in spot filter SELEX, blocking agents were added as described in Materials and Methods.

RNA pool binding with and without competitors heparin and tRNA. To evaluate SELEX progress, binding curves with purified HGF protein were routinely done with evolved pools during the course of these experiments. Representative binding curves are shown for HGF SELEX experiments 1 and 2 (Figure 3). These data were used to ascertain when SELEX was complete in that further progress was not likely to occur by performing additional rounds. HGF SELEX 1 reached its maximal binding by round 8, with a binding affinity of approximately 0.1 nM (Figure 3A; earlier rounds and round 9 were examined in other experiments). HGF SELEX 2 reached its maximal binding by round 10, with a binding affinity of approximately 0.1 nM (Figure 3B). HGF SELEX 3 reached its maximal binding by round 11, after seven rounds of spot filter partitioning followed by four rounds of conventional filter SELEX (see Figure 4B). A SELEX experiment which was deemed complete was characterized by cloning and sequencing (see below).

HGF, like other proteins which have large clusters of positively charged amino acids, exhibits a high degree of non-specific binding to polyanionic compounds. For example, random RNA pools bind to HGF with low nanomolar affinity, similar to the value reported for HGF binding to heparin, a polyanionic sulfated polysaccharide known to have an important biological role in HGF function (Zioncheck, Richardson *et al.* (1995) J Biol Chem. 270:16871-8). Competition binding to heparin as well as the non-specific competitor tRNA was done to provide an additional means of evaluating SELEX progress. This was done because the binding of random and evolved RNA pools to HGF occurs in a high-affinity range that makes it difficult to monitor progress. In other words, random

binding based on increased resistance to heparin competition. These data contributed to the choice of round 11 as the maximally binding pool from which we cloned and sequenced.

15 A similar, but more pronounced, effect was observed with tRNA as the competitor (Figure 5A). These data indicate that the round 11 pool from HGF SELEX 3 are at least four orders of magnitude more resistant to competition for binding to HGF than is random RNA. From these curves, it was determined that 800 nM tRNA is the maximum concentration at which complete binding of evolved RNA persists. Therefore, binding curves were done at this tRNA concentration to compare the binding of different evolved  
20 pools (Figure 5B). These curves were useful in determining that further SELEX rounds beyond round 11 did not improve binding.

Typical data from a similar set of binding competition experiments done for latter rounds of HGF SELEX 1 are summarized in Table 1.

Cloning and sequence analysis of HGF SELEXes 1, 2 and 3. Following  
25 determination of pool binding affinities for HGF, the optimal SELEX pools were cloned and sequenced in order to isolate and characterize individual aptamers. Data from 30N7 HGF SELEX 1 and 3 are summarized in Table 2, including binding affinities for many of the aptamers. A similar data set was generated for 30N8 HGF SELEX 2 (Table 3).  
Sequences from HGF SELEX 1, 2 and 3 are designated 8-seq. number, 10-seq. number,  
30 and 11-seq. number, respectively, referring to the total number of SELEX rounds each cloned pool was subjected to. Sequences were analyzed and organized into groups with

significant homology. Motifs were analyzed and predicted structures were drawn in order to analyze key features responsible for binding to HGF.

Inhibition of HGF-mediated stimulation of cell proliferation. HGF, while not a potent mitogen, does stimulate moderate proliferation of many cell lines, which can be measured by incorporation of  $^3\text{H}$ -thymidine. The inhibitory activity of HGF aptamers was assayed by measuring their effect on proliferation of human umbilical vein endothelial cells (HUVECs), or monkey bronchial epithelial (4MBr-5) cells. Based on the binding data and sequence family analysis, fourteen aptamers were chosen for analysis *in vitro* because they bind to HGF with high affinity and are representative of different sequence families. The sequences are shown in Table 4, aligned by a rough consensus that contains bases in common to several families. All sequences are 30N7 except 10-2 which is 30N8.

HGF stimulates proliferation of HUVECs by about two-to-three-fold (data not shown). The initial experiment indicated that aptamers 8-17, 8-102, 8-104, 8-122, 8-126, 10-2 and 11-208 were effective inhibitors of HGF-induced HUVEC proliferation with  $K_i$  values in the low nanomolar range (Figure 6). Aptamers 8-113 and 11-222 were less effective and 8-151 exhibited little or no concentration-dependent inhibition. The latter observation is consistent with the fact that aptamer 8-151 does not bind HGF with high affinity and actually binds worse than the random pool.

The following approaches were taken to reduce the length of aptamers which retained significant inhibition of HGF: 1) boundary determinations by biochemical separation of partially hydrolyzed aptamers; 2) sequence motif analysis and educated guessing; and 3) truncate SELEX.

Boundaries and truncation. Boundary determinations were done for a subset of aptamers that demonstrated *in vitro* inhibition of HGF activity. Using a standard alkaline hydrolysis procedure with 5'-end-labeled RNA, the 3'-boundaries of aptamers 8-17, 8-102, 8-104, 8-126, 10-1 and 10-2 were examined. Additionally, 3'-end-labeled RNA was used for 5'-boundary experiments with aptamers 8-17 and 8-102. These experiments were mostly uninformative, probably because the high degree of non-specific binding of RNA fragments, regardless of size, obscured the binding of truncated high-affinity aptamers to HGF. Non-specific binding of virtually all fragments gave no boundary information, and reducing the protein concentration did not help. Instead, we tried to use polyanionic

competitors tRNA and heparin to eliminate nonspecific binding to reveal the actual boundaries. The competitors reduced non-specific binding, and HGF was predominantly bound only by full-length aptamers, revealing no boundary information beyond the possibility that full-length aptamers are strongly preferred.

5       The sole exception was aptamer 8-102 which had a plausible 3'-boundary between two possible endpoints which made sense with respect to computer-predicted structures (Figure 7A). Based on the boundary data and structural data, two truncates of aptamer 8-102 were synthesized and analyzed for binding to HGF. The sequence of the full-length aptamer and the two truncates are shown, with fixed regions underlined:

10   gggaggacgaugcggcgagugccuguuuauucauguccgucgucagacgacugcccca (8-102) (SEQ ID NO:12)

ggacgaugcggcgagugccuguuuauucaugucc (36mer) (SEQ ID NO:13)

gacgaugcggcgagugccuguuuauuguc (28mer) (SEQ ID NO:14)

15       In binding to HGF, the 36mer bound almost as well as the full-length aptamer, while the 28mer bound no better than random 30N7 (Figure 7B), suggesting that the boundary data were correct.

Truncation by sequence structure prediction. Several attempts were made to base truncation on motif analysis and predicted structures, but these did not succeed in producing truncates which retained binding to HGF. For example, aptamer 8-17 folded  
20   into a reasonable predicted structure which suggested two obvious points of truncation from its 3' terminus, into a 38mer or 28mer (Figure 8A). However, binding analysis revealed that neither of these truncates retained significant binding to HGF (Figure 8B). These data suggest either that the predicted structure is incorrect or that some of the 3' region past base 38 is critical for high-affinity binding of aptamer 8-17 to HGF. These two  
25   hypotheses are not mutually exclusive. Nevertheless, we did not succeed in obtaining a useful truncate of 8-17 by boundary and structural prediction.

Truncate SELEX. In order to generate additional short aptamers, advanced rounds of the earlier SELEX experiments were subjected to additional rounds of truncate SELEX, using the Truncation SELEX method described in U.S. Patent Application Serial No.  
30   09/275,850, filed March 24 1999, entitled "Truncation SELEX Method," incorporated herein by reference in its entirety. Binding of RNaseH cleaved pools was examined to



determine which were the appropriate rounds to use to initiate truncate SELEX (data not shown). None of the RNaseH-cleaved evolved pools was clearly superior to another in binding to HGF, therefore, the pools which had been previously cloned were chosen to use in truncate SELEX. The encouraging result from this experiment was that after RNaseH  
5 treatment, the evolved pools bound better to HGF than did random RNA, suggesting that even in the absence of the fixed regions, significant binding affinity was retained. This observation was sufficient evidence to suggest that truncate SELEX could enrich for sequences that bound to HGF in the absence of fixed regions.

Three rounds of hybridization truncate SELEX were done in parallel, using as  
10 starting pools HGF SELEX 1 round 8 and HGF SELEX 3 round 11. The truncate SELEX rounds were done at equi-molar RNA and protein, starting at 1 nM and decreasing to 0.5 and 0.1 nM. Signal-to-noise ratios were very high during selection. Subsequent manipulations were satisfactory even though the amount of recovered RNA was sub-picomolar.

15 To evaluate the progress of the SELEX, binding affinities of truncate rounds two and three were determined and compared to those of the RNaseH-cleaved starting pools (Figure 9). For both SELEXes, the third round pools bound with improved affinity for HGF compared with the earlier rounds. Interestingly, the second rounds did not bind HGF better than the starting material. The dissociation constants for the third round truncate  
20 SELEX pools are 1-2 nM, representing a 2-3 fold improvement. While the magnitude of this improvement is not large, it is probably significant since HGF as a target did not easily yield affinity enrichment, probably because of its intrinsically high affinity for RNA.

The two pools were cloned and sequenced, and binding affinities were determined  
25 (Table 5). The truncated aptamer with the best binding affinity, Tr51, is among several sequences which are novel, that is, they were not found in the clones sequenced from the full-length SELEX pools. The emergence of novel sequences suggests that the truncate SELEX succeeded in amplifying aptamers that were relatively rare in the full-length pools. Aptamer Tr51 appeared more frequently than any other sequence, consistent with the  
30 observation that it has better binding affinity than any other truncate. Other sequences that

appeared multiple times also tend to be those with binding affinities near or better than the pool  $K_d$  of 1-2 nM.

HGF inhibition by the 36mer aptamer modified with 40K-PEG. The 36mer derivative of aptamer 8-102, described above, was tested for inhibition *in vitro* in a 4MBr-5 cell proliferation assay (Figure 10). Although the 36mer retained high-affinity binding to HGF, it did not retain inhibitory activity *in vitro* comparable to its parent aptamer 8-102 and aptamer 8-17 (Figure 10).

In order to improve the activity of the 36mer, it was tested in a formulation with a 3'-dT cap and 5'-40K PEG. The modified aptamer, designated NX22354, was tested for inhibition of HGF-mediated proliferation 4MBr-5 cells (Figure 11A). The data indicate that the 36mer-PEG aptamer inhibits HGF, and that it performs at least as well as the full-length aptamer 8-17, which had previously exhibited the strongest inhibition of all aptamers tested. As expected, the non-PEGylated 36mer did not inhibit HGF, suggesting that the addition of PEG and/or the 3'-cap contribute to the aptamer's bioactivity. This experiment was also done at lower aptamer concentrations, supporting the previous result and showing more clearly that the 36mer-PEG aptamer is a better inhibitor than the 8-17 full-length aptamer (Figure 11B). Also tested by this assay was a non-binding aptamer containing a 3'-dT cap and 5'-40K PEG, the VEGF aptamer NX1838, which had no effect on HGF stimulation (Figure 12). In this same experiment, a non-PEGylated version of NX1838 and the truncate SELEX aptamer Tr51 were shown to have no inhibitory effect on HGF (Figure 12). This suggests that Tr51, similar to the 36mer base aptamer of NX22354, may require 5'-40K-PEG to inhibit HGF function.

Inhibition of HGF-mediated stimulation of cell migration. HGF readily stimulates cell movement, hence the name, scatter factor. The inhibitory effect of HGF aptamers was assayed by measuring their effect on A549 cell migration across a Matrigel coated membrane with 8.0 micron pores as described in Materials and Methods (Table 6). The NX22354 aptamer fully inhibited HGF-mediated migration at both 1 and 0.2  $\mu$ M concentrations, but at 0.04  $\mu$ M, the effect was negligible. The monoclonal antibody control (sample 3) was moderately effective at the 1  $\mu$ g/mL dose, which is above its published  $EC_{50}$  value of 0.1-0.3  $\mu$ g/mL for inhibition of 4MBr-5 cell proliferation.

Combined inhibitory effect of HGF and VEGF aptamers on HUVEC proliferation.

It was reported that VEGF and HGF have an additive stimulatory effect on HUVEC proliferation (Van Belle (1998) Circulation. 97:381-90). This effect was observed when VEGF and HGF were added, singly and in combination, to HUVECs, and incorporation of <sup>3</sup>H-thymidine was measured (Figure 13). As expected, stimulation by HGF was relatively weak compared with that of VEGF and together, the stimulatory effect was greater than that elicited by VEGF alone.

Based on these curves, we chose to add each cytokine at 10 ng/mL for optimal stimulation in the aptamer inhibition experiments. The effect of adding one or both aptamers to the doubly-stimulated cells in the presence of both growth factors was then tested (Figure 14A). It was observed that each aptamer partially inhibits the stimulation and that both aptamers result in complete inhibition. Interestingly, the magnitude of the inhibitory effect of each aptamer roughly corresponds with the magnitude of the stimulation conferred by each cytokine. This observation suggests that the stimulatory effect of each cytokine can be inhibited independently, and that the two cytokines stimulate HUVECs independently.

Figures 14B and C depict controls in which each cytokine was administered separately, demonstrating that the HGF and VEGF aptamers do not cross-react, that is, each aptamer affects only the cytokine against which it was selected. For the HGF stimulated cells, inhibition by the HGF aptamer NX22354 was observed, but not by the VEGF aptamer NX1838 (Figure 14B). Conversely, stimulation by VEGF was inhibited by the VEGF aptamer NX1838, but was unaffected by the HGF aptamer NX22354 (Figure 14C).

These data, along with the fact that HGF, like VEGF, is an angiogenesis factor make it intriguing to consider dual administration of VEGF and HGF aptamers to treat tumors. Furthermore, the availability of aptamers which inhibit other growth factors suggests further combinations of the VEGF or the HGF aptamer in combination with other aptamers, for example, aptamers that inhibit bFGF, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF), keratinocyte growth factor (KGF), and/or their receptors allowing for the possibility that any combination of these inhibitors may be

omals, and a related section. The four partially substituted oligonucleotides were synthesized with a 1:1 ratio of 2'-OMe amidite:2'-OH amidite (Table 7). The data analysis measures the ratios of the selected to unselected RNA at each substituted purine position, based on quantitation of bands from the gel. The data are summarized by position (Figure 15). At each position, the three unsubstituted aptamers provide an important comparison, which is expressed as an average of the three unsubstituted aptamers with standard deviation represented by the error bars. Points that occur at ratios higher than that of the nearby positions are likely to require 2'-OH for binding.

15       The data strongly indicate that two positions, G5 and A25, do not tolerate 2'-OMe substitution. Two other positions, A3 and G10, show a slight preference above the standard deviation of the unselected RNA.

          The set of OMe aptamers were also examined for binding to HGF (data not shown). The binding data indicate that the OMe1 and OMe3 bind as well as the parent unsubstituted 36mer, whereas OMe2 and OMe4 bind do not bind as well. This suggests that the substitutions in OMe2 and OMe4 are less well tolerated with respect to HGF binding in solution, consistent with the fact that OMe2 and OMe4 are substituted at A25 and G5, respectively.

          To confirm these results, two aptamers were synthesized which are fully 2'-OMe substituted at the apparently well-tolerated positions. The sequences are shown below, with the 2'-OH-purines shown underlined. All other purines have 2'-OMe and the pyrimidines are 2'-fluoro substituted.

4x Sub 2'-OH. GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCC  
(SEQ ID NO:186)

30       2x Sub 2'-OH. GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCC  
(SEQ ID NO:187)

Sequence 4x Sub 2'-OH contains all four of the 2'-OH-purines in question, while 2x Sub 2'-OH has only the two 2'-OH-purines most likely to be required.

Binding of these oligomers to HGF was examined compared to the unsubstituted parent and the fully 2'-OMe substituted RNA (Figure 16). Based on these binding curves, NX22354 tolerates 2'-OMe substitution at all purines except G5 and A25 (aptamer 2x Sub 2'-OH) with minimal loss of binding affinity. The other two positions in question apparently are not required to be 2'-OH since aptamer 4x Sub 2'-OH binds no better than aptamer 2x Sub 2'-OH.

Two aptamers have been synthesized with 5'-40K-PEG and a 3'-dT cap: one is fully 2'-OMe substituted and the other contains 2'-OH at positions G5 and A25. One of these will presumably supplant NX22354 as the lead HGF aptamer for further testing *in vitro* and *in vivo*.

### Example 3. Results - c-met

c-met SELEX. In the c-met plate SELEX experiments, the concentration of nucleic acids was lowered initially, but then raised in later rounds so that the ratio of the nucleic acid to protein would be very high. This was done in order to create conditions of high stringency which may select for higher affinity aptamers. Stringency was also applied by increasing the number of washes.

SELEX pool binding. Binding of SELEX pools to c-met was assessed through round 7 (Figure 17). The binding data indicate that the SELEX resulted in about a 20 fold improvement in  $K_d$  from 20 nM to 1 nM for both (40N7) (Figure 17A) and (30N8) (Figure 17B) pools.

Since the c-met protein used in SELEX is an IgG fusion protein, random 40N7 and round 7c RNA pools, were tested for binding to human IgG<sub>1</sub> and c-met. The binding dissociation constants are set forth in Table 8. The affinity of round 7c RNA for both IgG<sub>1</sub> and c-met proteins improved about 50-fold. There are several interpretations of this result. Aptamers may have been selected which bind with better affinity to both proteins. This assumes that the difference in binding between IgG<sub>1</sub> and c-met is due to c-met specific aptamers. However, the two proteins were made in different cell lines that may have different glycosylation patterns which could influence binding. Thus, if the differences in

affinity are due to differences between the free IgG<sub>1</sub> protein and the IgG<sub>1</sub> domain in c-met, then there might be few if any c-met specific aptamers in the round 7 pool.

In order to address these issues further, random and round 5 RNA pools from both libraries were examined for binding to the c-met and KDR proteins (Figure 18). Both of these proteins were made in the same cell line and contain the same IgG<sub>1</sub>-His<sub>6</sub> sequence. Random RNA from both libraries binds about the same to each protein ( $K_d = \sim 50$  nM). Round 5 from both libraries of c-met SELEX binds better to c-met than to KDR ( $\sim 100$ -fold better for the 30N8 pool and 3-fold better for the 40N7 pool). However, round 5 RNA pools do bind better than random RNA to KDR. These results imply that, while there are probably aptamers that bind to human IgG<sub>1</sub> or (HIS)<sub>6</sub> tag in the round 5 pools, there may also be c-met aptamers.

Detection of IgG aptamers by PCR. Another approach for determining if IgG<sub>1</sub> aptamers are present in the SELEX pools was to subject them to PCR. Predominant IgG<sub>1</sub> aptamers have been isolated from N7 type libraries that have a known sequence (Nikos Pagratis and Chinh Dang, personal communication). For the PCR, a DNA oligonucleotide:

ML-124; 5'-ACGAGTTTATCGAAAAAGAACGATGGTTCCAATGGAGCA-3' (SEQ ID NO:188), was used that is complementary to the most prevalent N7-series human IgG<sub>1</sub> aptamer sequence, and differs by only a few bases from most other IgG<sub>1</sub> aptamers. This PCR primer is the same length as the selected sequence of the major IgG<sub>1</sub> so that it can tolerate mismatches and hybridize to similar sequences.

The ML-124 3'-primer: ML-34; 5'-CGCAGGATCCTAATACGACTCACTATA-3' (SEQ ID NO:189), was used with a 5'-primer containing the T7-promoter sequence present in all cloned aptamers to amplify 40N7 series nucleic acids pools: random, 1a, 2a, 3a and 4a (data not shown). Since IgG<sub>1</sub> aptamers have not been isolated from an N8 type library, this analysis was not done for the 30N8 SELEX. PCR of random and c-met SELEX round 1a pools yielded no signal after 20 cycles. However, rounds 2a, 3a, and 4a had steadily increasing signals that were easily detectable after 10 PCR cycles. Thus IgG<sub>1</sub> aptamers appeared relatively early in the 40N7 SELEX experiment. For a negative control, PCR was done with a nucleic acid pool from a

SELEX known to lack IgG<sub>1</sub> aptamers. For positive controls, PCR was done with pools from either an N7-based IgG<sub>1</sub> or CTLA4-IgG<sub>1</sub> SELEX. IgG<sub>1</sub> aptamers were first isolated from both of these SELEXes. The negative control had no detectable IgG<sub>1</sub> aptamers after 20 PCR cycles. The positive controls had detectable signals after 10 PCR cycles.

C-met aptamers. The sequences of 19 clones from round 7c-40N7 fall into five families with two sequences each, a group with three unrelated members, and six sequences closely related to known IgG<sub>1</sub> aptamer sequences (Table 9). Thus, at least 6 of the 19 clones (32 %) are human IgG<sub>1</sub> aptamers. This confirms the results of previous analysis that indicated the presence of IgG<sub>1</sub> aptamers in this SELEX experiment.

Of the 13 clones sequenced from round 7b-30N8, six are almost identical, another five are closely related, and two are distinct (Table 10).

Nine clones were tested for binding to c-met or KDR, six from the 40N7 series and three from the 30N8 series. These clones were chosen for the following reasons. Clone 7b-4 is the most frequent clone in Family 1 and is representative of almost all of the sequences isolated from the 7b-30N8 library. Clones 7b-10 and 7b-12 are the two clones from the 7b-30N8 library that had different sequences. From the 7c-40N7 pool, the chosen representatives were: Family 1 (clone 7c-1); Family 2 (clone 7c-4); Family 3 (clone 7c-23); Family 4 (clone 7c-26); Family 5 (clone 7c-25); and the presumed IgG<sub>1</sub> Family (clone 7c-3).

Results are shown for only two clones, including 7c-1, which was the only one observed to bind to c-met better than KDR (Figure 19A). Clone 7c-1, which appeared twice in the 40N7 series, may exhibit biphasic binding behavior with a high affinity binding  $K_d$  of ~50 pM and a lower affinity binding  $K_d$  of ~5 nM. All eight other clones bound to KDR as well as to c-met, including 7c-3, which is shown here as representative example (Figure 19B). Clone 7c-3 and all others besides 7c-1 are presumed to be IgG<sub>1</sub> aptamers.

In summary, two clones (identical to 7c-1) out of 32 apparently bind c-met specifically and with high affinity. The remaining clones appear to be IgG<sub>1</sub> aptamers.

#### Example 4. Isolation of integrins and integrin ligands

$\alpha_v\beta_3$  integrin was isolated from human placenta and purified by immunoaffinity chromatography essentially as described by (Smith and Cheresch (1988) J. Biol. Chem. 263:18726-31). In brief, human placentas were diced and the tissue fragments were extracted in a buffer containing 100 mM octyl- $\beta$ -D-glucopyranoside detergent (Calbiochem, San Diego, CA). The extract was cleared by centrifugation and applied to an immunoaffinity column ( $\alpha_v\beta_3$ -specific monoclonal antibody LM609 affixed to Affi-Gel 10, (Chemicon International, Inc., Temecula, CA)). Protein bound to the column was eluted with a low-pH buffer and fractions were immediately neutralized and analyzed for integrin content by SDS-polyacrylamide gel electrophoresis. Integrin-containing fractions were pooled and aliquots of the purified material were stored at  $-80^\circ\text{C}$ . Purified human  $\alpha_v\beta_3$  was also purchased from Chemicon International, Inc, as was human  $\alpha_v\beta_3$  integrin.  $\alpha_{\text{IIb}}\beta$  and fibrinogen were purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). Vitronectin was purified from outdated human plasma according to the procedure of (Yatohgo *et al.* (1988) Cell Struct. Func. 13:281-92), using heparin affinity chromatography.

### Example 5. Generation of nucleic acid ligands to integrins

**A DNA template library of sequence:**

20    5'-*ttatacgactcactatagggagacaagaataaacgctca*  
nnnnnnnttcgacaggaggctcacacaggc-3' (SEQ ID NO:190)

was prepared by chemical synthesis. The italicized nucleotides correspond to a T7 RNA polymerase promoter. There are 40N residues (a, g, t or c). A short DNA primer "3N8": 5'- gcctgttgtagcctcctgtcgaa-3' (SEQ ID NO:191)

25 was annealed to the template and extended using Klenow DNA polymerase (New England Biolabs, Beverly, MA). The double-stranded DNA product served as a product for T7 RNA polymerase transcription (enzyme obtained from Enzyco, Inc., Denver, CO) to generate a library of random-sequence RNAs. 2'-fluoro-CTP and -UTP were used in place of the 2'-OH-pyrimidines.

30 For application of the SELEX process to  $\alpha_v\beta_3$  integrin, the purified protein was diluted 1000-fold from detergent-containing storage buffer into 50 mM MES (2-[N-



morpholino]ethanesulfonic acid), pH 6.1, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, to a final concentration of approximately 0.2 µg/mL. 3.2 µ polystyrene particles (IDEXX Laboratories, Inc., Westbrook, ME) were added to the diluted protein and the mixture was rotated overnight at 4°C. The beads were collected by centrifugation and blocked by incubation in 3% BSA in MES buffer (above) for one hour at room temperature. Blocked beads were washed several times by resuspension in binding buffer (50 mM Tris-HCl, pH 7.4 (at 37°C), 145 mM NaCl, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 0.1 mM MnCl<sub>2</sub>, 0.01% BSA). For one round of selection, integrin-coated beads were mixed with RNA and rotated at 37°C for 4 hours to allow equilibration of the RNA with the immobilized protein. The beads were then collected by centrifugation and washed at least 5 times in binding buffer by rapid resuspension and pelleting, without additional incubation. RNAs that remained bound to the beads were eluted overnight at 37°C in binding buffer plus 100 µM cyclic RGD peptide ("cRGD") (GPenGRGDSPCA, Life Technologies, Gibco BRL, Gaithersburg, MD). Eluted RNAs were extracted with phenol, then chloroform:isoamyl alcohol (24:1), and ethanol precipitated. The RNA pellet was resuspended and annealed to primer 3N8 for reverse transcription using avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL). The cDNA pool was amplified by the polymerase chain reaction using the 3N8 primer and primer "5N8":  
5'-taatacgactcactatagggagacaagaataaacgctcaa-3' (SEQ ID NO:192)  
and T. aquaticus DNA polymerase (Perkin Elmer-Cetus, Foster City, CA). Transcription of the PCR product generated an RNA pool to initiate a new round of selection. For the first round of selection 1 nmol of RNA (approximately 6 x 10<sup>14</sup> sequences) was incubated at 2 µM concentration with a volume of bead suspension equivalent to 50 pmol of protein (assuming all the integrin had adsorbed to the beads). In subsequent rounds, the concentration of RNA and protein-coated beads were both reduced to demand higher affinity binding interactions.

The affinity of individual RNAs and RNA pools for  $\alpha_v\beta_3$  was determined by titration of biotinylated RNA with a small quantity of immobilized integrin. Bound RNA was detected through the biotin moiety. Biotinylated RNA was prepared according to standard transcription protocols, but including a 5-fold molar excess of a 5'-biotin-modified GMP over GTP in the reaction mixture. Methods for synthesizing 5'-biotin-modified guanosine

nucleotides are described in WO 98/30720 entitled "Bioconjugation of Oligonucleotides," specifically incorporated herein by reference in its entirety. The modified nucleotide is incorporated at the 5' end of the transcript in proportion to its representation in the guanosine pool. 96-well microtiter plates (Immulon 2, Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight at 4°C with 100  $\mu$ L purified  $\alpha_v\beta_3$  at a concentration of 0.25  $\mu$ g/mL in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM  $MgCl_2$ , 2 mM  $CaCl_2$ , 0.1 mM  $MnCl_2$ . Coating concentrations were 0.8  $\mu$ g/mL for  $\alpha_{Ib}\beta_3$  and 0.3  $\mu$ g/mL for  $\alpha_v\beta_3$ . Wells were blocked with 200  $\mu$ L of a solution of 3% BSA in the same buffer (1 hour at room temperature) then rinsed 3 times with 200  $\mu$ L binding buffer (50 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1 mM  $MgCl_2$ , 2 mM  $CaCl_2$ , 0.1 mM  $MnCl_2$ , 0.1% BSA). Individual RNAs or RNA pools were denatured briefly at 93°C in binding buffer without divalent cations or BSA, then serially diluted in the same buffer. 50  $\mu$ L binding buffer containing 2X-concentrations of divalent cations and BSA were added to each well, followed by 50  $\mu$ L RNA dilution.

15 RNAs were allowed to incubate in the integrin-coated wells at 37°C for 30-60 minutes. Unbound RNAs were removed by 3 rapid washes in binding buffer. To detect bound RNA, 100  $\mu$ L of a 1:2500 dilution in binding buffer of streptavidin-alkaline phosphatase conjugate (Calbiochem) were incubated in each well for 30 minutes at room temperature, followed by three rapid washes, as above. 100  $\mu$ L/well p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) was added and incubated at room temperature for 30 minutes. Color development was monitored by absorbance at 405 nm. Binding data were fit to an equation that describes the fraction of RNA or protein bound as a function of  $K_D$ , and the total concentrations of RNA and protein in the binding reaction for both monophasic and biphasic binding behavior (Green *et al.* (1996) Biochem. 25 35:14413-24). A control RNA corresponding to a sequence-scrambled version of aptamer 7.24:

5'-gggagacaagaauaaucgcuaacguugaugcugcauuaggagauugaccgcuaucuccuucgacaggaggcucacaacaggc-3' (SEQ ID NO:193)

was used to monitor non-specific binding of RNA under the conditions of the assay.

30 After seven rounds of the SELEX process, the amount of RNA specifically bound to the integrin-coated beads had increased substantially (data not shown). Although

immobilized  $\alpha_v\beta_3$  showed no detectable affinity for random sequence RNA, the round 7 RNA pool bound with an equilibrium dissociation constant ( $K_D$ ) of approximately  $4 \times 10^{-7}$  M (Figure 20). The round 7 affinity-enriched pool was cloned and sequences were determined for individual molecules in the mixture. Of 92 sequences obtained, 35 (38%) were very highly related to one another, in many cases differing at no more than a single base position. These sequences are collectively referred to as "Family 1." It is likely that many if not most of these RNAs derived from a single precursor as a result of errors in replication during the RT and PCR steps. Another 25 sequences (27%) shared a short motif (CCUGCC) that defined a second sequence family ("Family 2"). The remaining 32 sequences (35%) were not obviously related to sequences in Families 1 or 2 and were thus termed "orphan" sequences. The large percentage of orphan sequences in the round 7 pool suggested that a great deal of sequence complexity remained in the population. Therefore, the SELEX process was continued in the hope of further enriching for high affinity sequences whose representation in the round 7 pool may have been low. Indeed, a substantial improvement in the affinity of the RNA pool was observed after 8 additional rounds of affinity selection (round 15, Figure 20). No further improvement was seen after two more rounds of selection (round 17, Figure 20), so clones were isolated from the round 15 and round 17 RNA pools and the sequences of individual isolates were compared to those obtained at round 7. Twenty-seven of 39 sequences derived from the round 15 pool (69%) were members of the highly conserved sequence family, Family 1. Three sequences (8%) could be grouped with Family 2 and 9 sequences (23%) were orphans. All of the 18 sequences isolated from the round 17 pool were members of sequence Family 1. Thus, in this case, additional rounds of the SELEX process served to focus the RNA population on a single high-affinity sequence family that was already predominate at round 7.

Table 11 shows the sequences of the major family of 2'-F-pyrimidine RNAs with high affinity for  $\alpha_v\beta_3$  (Family 1). Clone names indicate the selected RNA pool from which each sequence was derived (round 7, round 15 or round 17) followed by a unique clone number. Note that in several cases identical sequences were isolated from different RNA pools; in these cases, both clone names are given. (Clones 17.12A and B were isolated as end-to-end inserts in a single plasmid.) Numbers in parentheses indicate the

frequency with which a particular sequence was isolated; if no number is given the clone was obtained only once from the selected RNA pool. Sequences of the 5' and 3' fixed sequence regions common to all of the clones are shown at the top in lower case letters. Gaps have been inserted into many of the sequences to highlight the strong sequence conservation among most of the clones. The length of the random sequence region is shown for each RNA, as well as an estimate of the  $K_D$  for binding to immobilized  $\alpha_v\beta_3$ , where it was determined (ND = not determined). The  $K_D$  value provided is generally based on one or the average of two determinations. Family 2 sequences isolated from the  $\alpha_v\beta_3$  SELEX are shown in Table 12. The short motif (CCUGCC) held in common among all the sequences is indicated in boldface letters. In Table 13, sequences with no obvious relationship to Families 1 or 2 are shown. Groups of similar sequences with only two (7.41 (SEQ ID NO:279) and 7.93 (SEQ ID NO:291)) or three (7.11 (SEQ ID NO:276), 7.82 (SEQ ID NO:288) and 7.101 (SEQ ID NO:294)) members are also included in Table 13.

The substantial affinity improvement between rounds 7 and 15 must be due in part to the loss of lower affinity species from the population; however, the introduction of and selection for higher affinity sequence variants of Family 1 may also have contributed to the overall affinity enrichment of the pool. While the affinity of relatively few sequences from the round 7 pool were measured, their affinities for immobilized  $\alpha_v\beta_3$  were generally less than that of RNAs derived from rounds 15 and 17 (Tables 11-13).

#### Example 6. Specificity of the nucleic acid ligands to integrins

In general, aptamers selected for high-affinity binding to a particular target protein show relatively weak binding to other related proteins, except in cases where the degree of homology is very high (for example, see (Green *et al.* (1996) *Biochem.* 35:14413-24; Ruckman *et al.* (1998) *J. Biol. Chem.* 273:20556-67)). Significant homology exists within the families of integrin alpha and beta sub-units, and both alpha and beta sub-units are shared among members of the integrin superfamily. Thus, it was of interest to assess the relative affinity of the  $\alpha_v\beta_3$  aptamers for closely related integrins. The affinities were determined using the methods described above. The Family 1 aptamer 17.16 (SEQ ID NO:249), was chosen as a representative of the major sequence family. Figure 21 shows

that aptamer 17.16 bound with identical affinity to purified, immobilized  $\alpha_v\beta_3$  and to the platelet integrin,  $\alpha_{IIb}\beta_3$ , in a 96-well plate binding assay. Although these two proteins share the  $\beta_3$  sub-unit in common, an alignment of the  $\alpha_v$  and  $\alpha_{IIb}$  amino acid sequences shows only 36% overall sequence identity (Fitzgerald *et al.* (1987) *Biochem.* 26:8158-65). Short stretches of exact sequence identity, 5 to 9 amino acids in length, do occur, primarily within four putative calcium-binding domains of each  $\alpha$  sub-unit. Binding of aptamer 17.16 to integrin  $\alpha_v\beta_3$  was also tested. The  $\beta_3$  sub-unit shares 56% sequence identity with  $\beta_3$  and is more closely related to  $\beta_3$  than other members of the beta sub-unit family (McLean *et al.* (1990) *J. Biol. Chem.* 265:17126-31; Suzuki *et al.* (1990) *Proc. Nat. Acad. Sci.* 87:5354-8). No aptamer binding to immobilized integrin  $\alpha_v\beta_3$  was observed (Figure 22), although an  $\alpha_v$ -specific antibody detected the presence of  $\alpha_v\beta_3$  protein adsorbed to the surface of the well (data not shown). Together, these data strongly suggest that aptamer 17.16, and by extension the other members of sequence Family 1, bind primarily to the  $\beta_3$  sub-unit of  $\alpha_v\beta_3$ . Furthermore, the high-affinity binding of the aptamer to the platelet integrin,  $\alpha_{IIb}\beta_3$ , extends its range of potential application to indications involving detection of platelets or inhibition of their function.

#### Example 7. Aptamer inhibition of ligand binding to purified integrins

While the SELEX process identifies RNA sequences with high affinity for a particular target, the procedure used in this example was designed to bias for the recovery of ligand binding site inhibitors by the inclusion of a cRGD peptide competitor in the elution buffer. To test whether aptamer 17.16 (SEQ ID NO:249) could block the ligand binding site of  $\alpha_v\beta_3$  or  $\alpha_{IIb}\beta_3$ , purified vitronectin and fibrinogen were biotinylated and incubated with one or both of the immobilized integrins in the presence or absence of varying concentrations of the aptamer or a non-binding control RNA. This was done as follows: purified integrin ligands, vitronectin and fibrinogen, were biotinylated according to (Smith *et al.* (1990) *J. Biol. Chem.* 265:12267-71). Briefly, proteins were dialyzed into 0.1 M  $\text{NaHCO}_3$  and 0.1 M NaCl. N-hydroxysuccinimido-LC-biotin (Pierce) was dissolved at 1 mg/mL in DMSO and added to the protein at a ratio of 0.1 mg biotin per 1 mg protein. The reaction was allowed to rotate at room temperature for 2 hours. Biotinylated proteins were dialyzed into phosphate-buffered saline and their concentrations determined by

220.507 24). The concentration of competitor that inhibited 50% of the maximum signal above background ( $IC_{50}$ ) was determined from the fitted curve.

Known ligand binding inhibitors, including an RGD peptide and the  $\alpha_v\beta_3$ -specific antibody LM609, were included as positive controls for the assay. Figure 23A shows inhibition of biotinylated vitronectin binding to immobilized  $\alpha_v\beta_3$ . Aptamer 17.16 inhibited the binding interaction with an  $IC_{50}$  of 4.7 nM while the control RNA showed no inhibition. By comparison, the  $IC_{50}$  of RGD peptide inhibition was 1.4 nM and that of LM609 was 2.7 nM. Unmodified vitronectin inhibited the binding of the biotinylated material with an  $IC_{50}$  of 59 nM. Similar data were obtained for aptamer inhibition of fibrinogen binding to  $\alpha_v\beta_3$  (Figure 23B) and for fibrinogen binding to  $\alpha_{IIb}\beta_3$  (Figure 23C).  $IC_{50}$  values for the data in Figure 23B were: 17.16, 9.5 nM; control RNA, not measurable; RGD peptide, 1.0 nM; LM609, 6.3 nM; unmodified fibrinogen, 43 nM.  $IC_{50}$  values for Figure 23C were: 17.16, 6.5 nM; control RNA, not measurable; RGD peptide, 21 nM; unmodified fibrinogen, 15 nM. Thus, aptamer 17.16 is an effective competitor of  $\beta_3$  integrin ligand binding and, on a molar basis, has an inhibitory potency nearly equivalent to that of a bivalent antibody.

#### Example 8. Nucleic acid ligand binding to human platelets

Aptamer 17.16 (SEQ ID NO:249) was selected for binding to purified human  $\alpha_v\beta_3$  adsorbed to the surface of a polystyrene bead. *In vitro* assays to measure the affinity of the

aptamer for purified  $\beta_3$  integrins were also done in the context of hydrophobically-adsorbed protein. Thus, an important test of aptamer function was to determine its capacity to bind to native protein on the surface of cells. Human platelets were chosen for this purpose because of their ease of isolation and their high level of expression of integrin  $\alpha_{IIb}\beta_3$ . Because  $\alpha_{IIb}\beta_3$  undergoes a conformational change upon platelet activation, binding of the aptamer to both resting and thrombin-activated platelets was tested. This was done as follow: fluorescein-conjugated RNA was prepared according to (Davis *et al.* (1998) Nuc. Acids Res. 26:3915-24). Briefly, RNA was transcribed in the presence of a 5-fold molar excess of the initiator nucleotide guanosine-5'-O-(2-thiodiphosphate) (Calciochem), followed by conjugation of the gel-purified RNA to 5-iodoacetamidofluorescein (Pierce, Rockford, IL). Platelet-rich plasma was prepared from freshly-drawn citrated human blood by centrifugation at 1000 rpm for 15 minutes in a table top centrifuge. For activated platelets, cells were incubated for 15 minutes at room temperature at  $2 \times 10^7$ /mL in calcium- and magnesium-free Dulbecco's PBS with 2.5 U/mL thrombin and 5 mM Gly-Pro-Arg-Pro (GPRP) to inhibit platelet aggregation. Cells were diluted 1:10 into binding buffer (20 mM HEPES, pH 7.5, 111 mM NaCl, 5 mM KCl, 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 0.1% BSA, 0.01%  $NaN_3$ ). Resting cells were diluted similarly, without exposure to thrombin or GPRP. The activation state of resting and thrombin-treated cells was monitored by staining with fluorophore-conjugated antibodies to CD61 ( $\beta_3$  integrin subunit), which binds to all platelets, and to CD62 (P-selectin), a marker of platelet activation. Antibodies were obtained from Becton-Dickinson Immunocytometry Systems, San Jose, CA. Fluorescein-conjugated RNAs were diluted in water to 4  $\mu$ M and denatured briefly at 93°C, then diluted to 2  $\mu$ M with 2X-concentrated binding buffer. RNAs were then serially diluted in binding buffer. Each dilution was mixed 1:1 with resting or activated platelets and allowed to incubate in the dark at room temperature for 30 minutes. The incubation mixtures were applied directly to a Becton Dickinson FACSCalibur flow cytometer to determine the mean fluorescence intensity of the sample. Under such equilibrium binding conditions, an estimate of the  $K_D$  for aptamer binding to the cell surface integrin could be obtained.

Non-specific RNA binding to platelets was measured using a control RNA of similar length and base composition to aptamer 17.16. Non-specific binding became significant at

concentrations above approximately 100 nM. Specific binding of the aptamer was distinguished from non-specific binding by the addition of 5 mM EDTA to the sample: EDTA had no effect on the binding of the control RNA but reduced aptamer binding to the level of the control. Specific binding of the aptamer was thus defined as the difference  
 5 between the fluorescence intensity of the sample before the addition of EDTA (specific + non-specific) and the fluorescence intensity after the addition of EDTA (non-specific only).

Figure 24 shows representative data for the EDTA-sensitive component of aptamer binding to both resting and thrombin-activated human platelets. The maximum binding  
 10 signal is approximately 2-fold higher to activated platelets, consistent with the slightly higher level of  $\alpha_{\text{IIb}}\beta_3$  on such cells (Wagner *et al.* (1996) Blood 88:907-14). However, the estimated  $K_D$  for aptamer binding to platelets was approximately 10 nM for both cell populations, equivalent to the value determined for binding *in vitro* to purified  $\alpha_{\text{IIb}}\beta_3$ . Furthermore, aptamer 17.16 binds to both resting and activated platelets with an affinity  
 15 equivalent to that reported for Reopro (abciximab, chimeric 7E3 Fab), an approved  $\alpha_{\text{IIb}}\beta_3$  antagonist (Mousa *et al.* (1998) J. Pharm. Exp. Ther. 286:1277-84).

#### Example 9. Nucleic acid ligand biodistribution in rabbit venous clot model

To explore the application of a  $\beta_3$ -specific aptamer in clot imaging, aptamer 17.16  
 20 was labeled at the 5' end with technetium-99m ( $^{99\text{m}}\text{Tc}$ ) and its biodistribution was monitored in a rabbit model of venous thrombosis. In this model, a clot is generated *in situ* in the isolated jugular vein of a rabbit from human platelet-rich plasma. Blood flow across the clot is re-established and the radiolabeled aptamer (or a non-binding control RNA) are introduced into the bloodstream via the ipsilateral ear vein. The distribution of  
 25 the radiolabel into various tissues is reported as the per cent of the injected dose per gram of tissue.

The experiment was performed as follows: Aptamer 17.16 and a control RNA of similar length and base composition were transcribed using a 5-fold molar excess of 5'-(O-hexylamino) guanosine monophosphate. Each RNA was conjugated to  $\text{Hi}_{15}$  at 50 mg/mL  
 30 aptamer in 30% dimethylformamide with 5 molar equivalents of  $\text{Hi}_{15}$ -NHS buffered in 100 mM NaBorate pH 9.3, for 30 minutes at room temperature. The conjugation reactions



were washed over a 30,000 molecular weight cut-off filter (Microcon 30, Amicon, Inc., Beverly, MA) to remove excess  $\text{Hi}_{15}$  cage. The RNAs were then labeled with  $^{99\text{m}}\text{Tc}$  in the following manner: to 1 nmol  $\text{Hi}_{15}$ -aptamer was added 200  $\mu\text{L}$  of 100 mM sodium phosphate buffer, pH 8.5, 23 mg/mL NaTartrate, and 50  $\mu\text{L}$  [ $^{99\text{m}}\text{Tc}$ ] pertechnetate (5.0 mCi) eluted from a  $^{99}\text{Mo}$  column (Syncor, Denver) within 12 hours prior to use. The labeling reaction was initiated by the addition of 10  $\mu\text{L}$  5 mg/mL  $\text{SnCl}_2$ . The reaction mixture was incubated for 15 minutes at  $90^\circ\text{C}$ . Unreacted  $^{99\text{m}}\text{Tc}$  was removed by spin dialysis through a 30,000 molecular weight cut-off membrane (Centrex, Schleicher & Schuell) with two 300  $\mu\text{L}$  washes. This labeling protocol results in 30-50% of the added  $^{99\text{m}}\text{Tc}$  being incorporated with a specific activity of 2-3 mCi/nmol RNA.

For biodistribution studies, rabbits were anesthetized with isoflurane. A two centimeter section of the right jugular vein was isolated *in situ* and all the branches were ligated. A catheter was inserted into the facial vein. The isolated vein segment was temporarily ligated above and below the catheter. The vein segment was flushed with saline. 1000 USP units of heparin was administered intravenously. 300-400  $\mu\text{L}$  of fresh human platelet-rich plasma (citrate) activated with calcium and thrombin was instilled into the isolated vein segment and allowed to clot. After 30 minutes the ligatures were removed and blood flow over the thrombus was re-established (confirmed by the injection of 200  $\mu\text{L}$  of air into the ipsilateral ear vein). [ $^{99\text{m}}\text{Tc}$ ]-conjugated aptamer or control RNA was injected into the ipsilateral ear vein. At 1 hour the rabbit was exsanguinated and tissues were weighed and counted in a Wallac 1470 gamma counter. The aptamer and control RNA were tested at 1 nmol/kg (approximately 0.03 mg/kg).

For aptamer 17.16, radiolabel accumulated in the clot to a significant degree by one hour after injection, while similar accumulation was not observed with the control RNA (Figure 25). Blood clearance of the radiolabel was apparently rapid and mediated primarily by a renal mechanism as judged by moderate accumulation of radioactivity in the kidney for both the aptamer and control RNA. Thus, aptamers specific for  $\alpha_{\text{IIb}}\beta_3$  or for other proteins expressed at high levels on the surface of platelets or within the matrix of a clot will serve as useful agents for rapid imaging of thrombi.

**Table 1.** Binding affinities of HGF SELEX 1 pools with and without competitor tRNA.

RNA pool	$K_d$ (nM)	$K_d$ (nM) w/ tRNA
Random 30N7	1.6	550
HGF SELEX 1 Rd.8	0.07	0.35
HGF SELEX 1 Rd.9	0.09	0.42

Table 2. HGF 30N7 aptamer sequences and binding affinities.

Seq. no. <sup>a</sup>	30N7 random region <sup>b</sup>	SEQ ID No:	K <sub>d</sub> (nM)
8-122 (2,1)	CGGUGUGAACCUGUUUAUGUCCGCGUACCC	18	0.097
8-108	CGGUGUGGACCUGUUUAUGUCCGCGUACCC	19	ND <sup>c</sup>
8-115	AGUGAUCCUAUUUAUGACAUCGCGGGCUGC	20	ND
8-125	UGUGAACCUGUUUAUGUCAUCUUUGUCGU	21	0.075
8-155 (1,1)	UGUGAACCUAUUUAUGCCAUCUCGAGUGCC	22	0.093
8-162	CGUGAGCCUAUUUAUGUCAUCAUGUCUGUC	23	ND
8-165	CGAGAGCCUAUUUAUGUCAUCAUGCCUGUG	24	0.100
8-171	CGGGAGCCUUUUUAUGUCAUCAUGUCUGUG	25	0.120
8-114 (4,2)	CGUGAGCCUAUUUAUGUCAUCAUGUCUGUG	26	0.071
8-203	CGCGAGCCUAUUUAUGUCAUCAUGUCUGUG	27	0.140
8-215	CGUGAGCCUAUUUAUGUCAUCAUGUCUGGU	28	0.077
8-217	CGUGAGCCUAUUUACGUCAUCAUGUCUGUG	29	ND
8-222	UGUGAACCUAUUUAUGCCAUAUUGUCUGUG	30	0.130
8-225	CGUGAGCCUAUUUAUGUCAUCAAGUCUGUG	31	ND
8-102	CGAGUGCCUGUUUAUGUCAUCGUCCGUCGU	12	0.060
11-9	CGUGAGCCUGUUUAUGACCUCGUCCAUGGC	32	0.074
11-58	CGUGAGCCUAUUUAUGACAUGUCCUCGAG	33	ND
11-59	CGUGAGCCUGUAUAUGUCAUUGUUCUCCGG	34	0.110
11-57	UGAGUACCUGUUUAUGUCACCACUUUCCCC	35	ND
11-103	UGAUUACCUA UUAUGUC UCGCCUCUC	36	0.200
11-110	UGAUUACCUAUUUUAUGUCAUGCUCUCCCC	37	0.086
11-65	UGAUAACCUGUUUAUGCCAUCGUGCUGGGC	38	0.110
11-167	UGAUAACCUGUUUAUGUCAUCGUGCUGGGC	39	ND
11-201	UGAGAACCUAUUUAUGUCAUCGUGCUGGGC	40	ND
11-162	UGAUAACCUAUUUAUGACGUCGUGGCUCUCC	41	ND
11-202	UGGGAACCUAUUUAUGUCAUC UCCGUCCC	42	ND
11-106	CGAUGAUGCCUGUUUAUGUCGAUGUCCCCC	43	0.120
11-158	CGAUAGCCUAUUUAUGACCUCGUCCCCGUG	44	0.170
11-112	CGUGAGCCUAUUUAUGACAUCGUUCUUGGC	45	ND
11-124	CGUGAGCCUAUCUAUGUCAUCGUGUGUGCC	46	ND
11-122	UGAGUACUAUUUAUGUCGUCGUUCGUGCC	47	ND
11-217	CGUGAGCCUCCAAUGACGUCGUCCUUGGC	48	0.071
8-104	GCGACUCAAUUCUGAAUCGUCUUGUCCCGUG	49	0.050
11-76	UCAGCGGCGCGAGCCUGUUUAUGUC UGCUG	50	0.076
"consensus"	<u>CGUGAGCCUAUUUAUGUCAUCGU-C-UG</u>	51	
11-8	UCAGUAUGACU UUUUAUGCA CGUUCGCCC	52	0.150
11-153	ACAGGUAGUCU UCUAUAGCA CUUCCUCCCC	53	0.190
11-157	UCAGAAUGACU UUCAUAGCA CGCUUCCCC	54	0.260
11-222	ACAUAAAGUCU UCUAUAGC UCGUCCUUUGUG	55	0.077
11-223	UCAGUAUGGCU UCUAUAGC UCGUCCUCGG	56	0.120
8-126 (3,1)	GUGACUAAAAUGGUGAUCCUCG UUUCCGC	57	0.099
8-101	GUGACUAAAAUGGUGAUCCUCGAUUUCCGC	58	0.095
8-105	GUGACUAAAAUGGUGAUCCUCGAUUGCCGC	59	ND
8-103	GCCGAAAAU UCGUCGACAUCUCCCUGUCUG	60	0.120

Table 2. (cont.)

Seq. no. <sup>a</sup>	30N7 random region <sup>b</sup>	SEQ ID NO:	K <sub>d</sub> (nM)
8-118	GGCGACUUUCCUCCAAUUCUCACCUCUGCA	61	0.160
8-119	GCCAUUCGAUCGA UUCUCCGCCGGAUCGUG	62	0.110
"consensus"	<u>CGUGAGCCUAUUUAUGUCAUCGU</u> -C-UG	51	
8-3 (2)	AUCCCGCGAC CAGGGCGUU UCUUCCUCGUCC	63	0.130
8-112 (3)	UCCCGAAUUAAGUGCGUU UCCUCCGCGUC	64	0.130
8-154 (3)	UCCCAAGAUUCAGGGCGUU UCUUCCUCGUCC	65	0.120
8-117	UCCCAAGUUUCAGGGCGUU UCUUCCUCGUCC	15	0.130
8-123	UCCCGAGUUUGAGGGCGUU UCUUCUUCGUCC	66	0.210
11-121	UCCAGUUUCAgGGGCGAU UCCUCUUCGUCC	67	ND <sup>c</sup>
8-17 (7,1)	GCGGCU CGAUG UCGUCUUAUCCCUUUGCCC	68	0.095
8-16	GCGGGCU CGAUG UCGUCUUAUCCCUUUGCCCC	69	ND
8-158	CCGGCU CGAUG UCGUCUUA CCCCUIUGCCC	70	0.310
11-104	GUUUGAG UGAUG UCGUCUUGUCCCGCCUGC	71	0.091
11-111	GUUAGAG UUUUG UCGUCUUGUCCCAUGUG	72	ND
11-163	GCUUGAGUC UUUG AUCGUCUUAUCCCUCCU	73	0.082
11-208	GUUUGAG UGACG AUCGUCUUGUCCCAUGUG	74	0.060
11-212	GUUUGAG UUAAA CAUCGGUUUUCUCCUG	75	0.075
11-6	GACGCG UUGAUU CAUCGUCUUAUCCUGCUG	76	0.240
11-126	GUUUGGGUCU UGAUC UCGUCUUGUCCCGUG	77	0.170
11-165	gUUGAUAGG AGUCAU CAUCGUCUUGUCCGC	78	0.073
11-215	GUAGUGAG UUUUCAUU GUCUUGUCCCGUG	79	0.091
11-151	UGAGUCAUAGUGUUG AUCGUCGUAUCCCGU	80	0.170
11-7	GUGGAGUCAA AUCGUCUUGUCCCUUGUCCU	81	0.110
11-166	GUUUGAG UUCUGACA CGUCUUGUCCCAUGC	82	0.079
11-17	GUUAGAGC GUGACAG UCGUCUUAUCCCGGGUCA	83	0.130
8-113 (2)	UGAAUCCUCUGGCUGAAAAU GACUUGUGC	84	0.083
8-60	UGAAUCCUUUGGCUGAAAAU GACUUGUGC	85	ND
11-221	GCAGAGCGAAAAUCGUCUUGUCCCGACGC	86	0.062
ORPHANS			
11-123	GUGACUAAAAUGGUGAUCCUCGUUUCGC	87	0.090
8-151	AGGACUAAUCCCUAAGGAAUAGCUUGCCCCG	88	8
8-174	UCGAGCUUCUGAGUUAAA CUGGGGCCUCCU	89	0.230
8-160	GUCCCCGAAUUUAAAGUGCGUUUCCUCCGGG	90	0.150
11-203	GGUUUUUCUUUUCUUGUUCUCUUUUUUCCCC	91	0.260
11-224	ACAGCGGCGACUAGCCUGUUCAUGCCUGCC	92	0.110
11-107	GUUCUGUGUGUCCACGUUCUUAUCCCUUGUG	93	0.140

**Table 2.** (cont.)

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<sup>a</sup>Clone series 8 is from HGF SELEX 1; series 11 is from HGF SELEX 3. Numbers in parentheses refer to repeat occurrences of the same exact sequence. For the series 8 clones, a second number refers to an exact match which was isolated in series 11.

<sup>b</sup>N7 fixed sequences are not shown. (5'-GGGAGGACGAUGCGG-N-CAGACGACUCGCCCGA-3')

<sup>c</sup>ND, not determined.

Table 3. HGF 30N8 aptamer sequences and binding affinities.

Seq. no. <sup>a</sup>	30N8 random region <sup>b</sup>	SEQ ID NO:	K <sub>d</sub> (nM)
10-28	CCUGUUCUGAAC GCAAAAUGGCGUGGUGGC	94	0.860
10-40	UGUCGUUAGUUUAUUGACAAGGCCCGAAG	95	0.350
10-52	UCUUAUUGUGUCCAGCUUCUCCUGCAGGC	96	0.160
10-72	UGUGGCAC UGUUGUCCACAAGGGCCUCA	97	0.450
10-8	UUGACAAGGUACCUGUUGCCUGGCGUUUCU	98	0.920
10-76	AGUUAGGCUUUAAGC ACG AUAUACAGCA	99	0.170
10-47	GUCAAGAGG AAAUGACACGG CUCCACUUUUA	100	0.390
10-2 (10)	GCCUGAGUUAACAUGACGG UUUGUGACCC	101	0.069
10-3	GCCUGAGUUAACAUGACGGUUUUGUGACCCCU	102	0.072
10-23 (4)	GUCUGAGUUGGACACAACGC AUUGAGACCC	103	0.330
10-24	GUCUGAGUUGGACACAACGC AUUGAGACCC	104	ND <sup>c</sup>
10-37	GUCUGAGUCCGU AGGGCGA UUUGUGUCCC	105	3.05
10-7	UGCCUUAAGAGCGGAA CUCCUGACCCACC	106	1.45
10-13	GAUCUGUUGGCGU GU CUACCCGACCCUCCU	107	0.720
10-17	AACCCUGUUGGCGU GA CGUCCCGACCCUCC	108	0.560
10-36	CGUUAGCAUCUGAACGAUGCCCAGCCUCAA	109	1.94
10-62	GUUAGACUCAACAUGAGUCCCAGCCUCAA	110	0.440
10-29	UCUGUUGGCGUCGU UCUCUGACCCUCCUC	111	1.75
10-48	GAGUCCCUGUUGAC UCGC UCUCUGACCC	112	0.310
10-16	UACAGCGUGUUGGUCCCGGACGGGACUUAU	113	0.210
10-11	CGCCUGGACCGUUGUUAUCCCCGUAGUC	114	0.610
10-18	CGUGAUUCCUACCAUCA GGUACCUAUCUUG	115	0.300
10-1 (2)	AGUGAUGUGAGAG CGUGCCUCUAGUCGGUG	116	0.094
10-57	CGAGCCUCCUACCGUUU AGGUACC AUCUUG	117	0.140
10-27	UUAGCCUCCGACCG UAA GGUCCUUUUCUUG	118	0.830
10-53	GGCCUCCAACCGCUAAA GGUUCCAUCUUG	119	0.310
10-49	CCCGACCUCCUGUAAACUGGUUGA GGCACUA	120	0.240
10-31 (2)	GGGUUCCUGAUUGACCCUGUCUCUAGACCC	121	1.90
10-58	GGGGAGGCCCUUCAGCCGUCUCCUUGACCC	122	0.440
10-63	UGUGAUGUGAGGGC GUGCUUCCUACGGUG	123	0.190
40N8 "hitchhiker" sequences			
10-19	UUCAUUAUGCAUCGAACAGUAUACCACAGGUGUUC AUGUG	124	ND
10-35	AUCCAAAUUCUGGUCAUGAGGCGUGCAGAUACUGCUGCG	125	2.33
10-38	UCUGCGGACGGUGAGGUUAAGUUGCAACGACUGCUUGGCG	126	7.38
10-42	CAGACCGUGCAAACCCCCUAGAGGGUUUUGUCAUUUAC	127	ND
10-56	CCUUAGGGCUCCAAAAUUCGGGCC CGUCGGGCCGAUCAC	128	0.280
10-68	CGCGGGAUUCUCUGAGGACGAGGCACGUGUGGGUAAUUCG	129	1.00
10-67	UCGGGCUUGGAUGUGGACGUGUAUUUCUAGCUGUGUACGC	130	0.640
10-4	UUGGGUCGGGACUCGAAAGGAUUUGAUAGGAUACAUGAAU	131	0.610

<sup>a</sup>Clone series 10 is from HGF SELEX 2. Numbers in parentheses refer to repeat occurrences of the same exact sequence.

<sup>b</sup>N8 fixed sequences are not shown. (5'-GGGAGAUAAAGAAUAAACGCUCAA-N-UUCGACAGGAGGCUCACAACAGGC-3')

<sup>c</sup>ND, not determined.

**Table 4.** List of HGF aptamers and their binding affinities which were tested *in vitro* for inhibition of activity.

Seq. No.	random region	$K_d$ (nM)	SEQ ID NO:
"consensus"	CGUGAGCCUAUUUAUGUCAUCGU-C-UG		
8-17	GCGGCU CGAUG UCGU CUUAUCCCUUGCCC	0.095	68
8-102	CGAGUGCCUGUUUAUGUCAUCGUCGUCGU	0.060	12
8-104	GCGACUCAAUCUGAAUCGUCUUGUCCCGUG	0.050	49
8-112	UCCCGAAUUUAAGUGCGUU UCCUCCGCGUC	0.130	64
8-113	UGAAUCCUCUGGCUGAAAUGA CUUGUGC	0.083	84
8-122	CGGUGUGAACCUGUUUAUGUCCGCGUACCC	0.097	18
8-126	GUGACUAAAUGGUGAUCCUCG UUUCCGC	0.099	57
11-8	UCAGUAUGACU UUUUAUGCA CGUUCGCCC	0.150	52
11-76	UCAGCGGCGCGAGCCUGUUUAUGUC UGCUG	0.076	50
11-166	GUUUGAG UUCUGACA CGUCU UGUCCCAUGC	0.079	82
11-208	GUUUGAG UGACG AUCGUCU UGUCCCAUGUG	0.060	74
11-222	ACAUAAGUCU UCUAUGC UCGUCCUUUGUG	0.077	55
10-2*	GCCUGAG UUAACAUGACG GUUUGUGACCC	0.069	101
8-151	AGGACUAAUCCCUAAGGAAUAGCUUGCCCG	8	88

\*10-2 contains N8 fixed sequences; all others are N7.

Table 5. HGF truncate SELEX 30N sequences.

Trunc Seq # <sup>a</sup>	# of hits	Sequence of random region (G)G-30N-CA	Identity to full- length <sup>b</sup>	K <sub>d</sub> (nM)	SEQ ID No:
		GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCC	NX22354	0.1	13
Tr7	(5)	CGGUGUGAACCUGUUUAUGUCCGCGUACCC	8-122	0.67	132
Tr45	(3)	UGGGAACCUAUUUUAUGUCAUCUCCGUCCC	11-202	1.7	133
Tr70		UGGGAACCUAUUUUAUGUCAUCGUCUGUGCC	New	2.4	134
Tr6		CGUGAGCCUAUUUAUGUCAUCAUGUCUGUG	8-114	9.0	135
Tr20		UGUGAACCUGUUUAUGCCAUCUCGAGUCCC	New	3.4	136
Tr23		UGUGAACCUAUUUAUGCCAUCUCGAGUGCC	8-155	ND <sup>c</sup>	137
Tr42		UGAUAACCUAUUUUAUGACGUCGUGGCUCCC	11-162	6.1	138
Tr44		AGUGAUCCUAUUUAUGCCGUCGCUUCUCGC	New	6.5	139
Tr65		AGAGNUCCUAUUUAUGACAUCCCAUGCCCC	New	1.4	140
Tr48		UGAUCACCUGUUUAUGCCAUCGUUCUGGGC	11-65	1.8	141
Tr28		GGUGACCCUUUUUAUGACAUCGCGUCUGGC	New	4.0	142
Tr51	(6)	AAUCACAGGAUCAACUUCUAUUCCCGCCC	New	0.06	143
Tr67		AAUCACAGGAUCGACUUUUUAUCCUGCCC	New	ND	144
Tr17		GC GGCUCGAUGUCGUCUUUAUCCCUUUGCCC	8-17	3.0	145
Tr27		UC GGCUCGUUGUCGUCUUUAUCCCUUUGCCC	New	ND	146
Tr18		GCUGGCUCGAUGUCAGGUUAUCCCUUUGCCC	New	ND	147
Tr4	(4,2) <sup>d</sup>	GUGACUAAAAUGGUGAUCCUCGUUCCGC	8-126	1.4	148
Tr31	(2)	UGAAUCCUCUGGCUGAAAAUGACUUGUGC	8-113	9.2	149
Tr15		GUUUGAGUGACGAUCGUCUUGUCCCAUGUG	11-208	8.8	150
Tr1		AUUGAUUCACUGCAUCCUUGACUCUUCUCCC	New	7.3	151
Tr5		CAGACGACUCGCCCCGAAGGACGAUGCGG	New	28	152
Tr14		GAGUUAUAUUUCGUCACCCGUUCCUUUGCCC	New	2.2	153
Tr59		ACAGUUUGUCUUCUAUAGCUCGUCGCCCC	New	7.2	154
Tr71		UCAGAAUGACUUUCAUAGCUCGCUUUCUCCC	New	7.7	155

<sup>a</sup>Tr1-36 and Tr37-72 clones are from series which were carried through 8 and 11 conventional rounds, respectively.

<sup>b</sup>Sequences indicated are identical to full length aptamers derived from series 8 or 11; NX22354 is a synthetic truncate based on boundary experiments, derived from sequence 8-102, shown here for comparative purposes.

<sup>c</sup>ND, not determined.

<sup>d</sup>(4,2) refers to 4 occurrences in the first series and two in the second series.



**Table 6.** Invasion of A549 cells through Matrigel is inhibited by HGF aptamer NX22354.

Sample	HGF 10 ng/mL	Inhibitor	Cells migrated
1	-	-	40
2	+	-	240
3	+	mAb <sup>a</sup> , 1 µg/mL	120
4	+	NX22354, 1 uM	40
5	+	NX22354, 0.2 uM	25
6	+	NX22354, 0.04 uM	200

<sup>a</sup>Anti-HGF antibody was MAB294 from R&D Systems, Inc.

**Table 7.** Partially 2'-O-methyl substituted variants of NX22354.

	SEQUENCE	SEQ. ID. No.
NX22354 (parent)	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCC *** ** * ** * * * * *	13
HGFOMe1	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCCg	156
HGFOMe2	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCCg	157
HGFOMe3	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCCg	158
HGFOMe4	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCCg	159

Parent 36mer sequence of NX22354 (purines marked with asterisks).

The substituted positions are indicated by underlines. The OMe1 sequence has five substitutions while the others have four. For technical reasons, a G residue was added at the 3'-end of each aptamer.

**Table 8.** Binding and Dissociation Constants

SELEX Round	Protein	$K_d$
random	IgG <sub>1</sub>	~1 µM
7c	IgG <sub>1</sub>	23 nM
random	c-met	100 nM
7c	c-met	2 nM

**Table 9.** 40N7 sequences isolated from a plate SELEX on human c-met.

<u>Clone name: (number of isolates).</u>	<u>Sequence<sup>a</sup></u>	<u>SEQ ID NO:</u>
<b>FAMILY 1:</b>		
7C -1: (2)	UUUGACUAUGUCUGACGGGUCUGUGGUCAAUUCGCCCC	160
<b>FAMILY 2</b>		
7C -4: (1)	AUCCGUGUUGAUGUCCAUAUAACCUUAUCCCGUCGCUCCC	161
7C -5: (1)	GUGUUGACUUCUAGCCAGAAUAACAUAUUUGUACCCCUCCC	162
<b>FAMILY 3</b>		
7C -2: (1)	UCGUUGAGCUUUUGAUAGGGCUUGUUCUUCGAGCGUCCC	163
7C-23: (1)	UGAUCUUGGGUUUGAUCGUAUAUACUUCACCCUCCGUCCC	164
<b>FAMILY 4</b>		
7C-26: (2)	CUCCUUUUCGCUAAACAAGACCACUUUGAGCCCUGCCCC	165
<b>FAMILY 5</b>		
7C-25: (1)	CCACCUCGUUACGUACUGAUUUUGGCAUCGCAGUUUGCCC	166
7C-27: (1)	GGGCACCUCGAUACGUACUGAUUUUGAAUAUCAGUUAGCCCC	167
<b>OTHERS</b>		
7C-21: (1)	CGAUUCGUCGUUAUAGAAAUGAUUUUGAAUGCACCUCUCCC	168
7C-24: (1)	UGUGUUUGUGUGUUGUGUUUGUUAUUCUGUUUGUGUCCU	169
7C-32: (1)	UCGGUCGUAAAAAUCGUUGGUGUCUAUCUAUUGUUCUCCC	170
<b>Presumed IgG<sub>1</sub> aptamers</b>		
7C -3: (1)	UGCUC CAGAGGAACCAUCGUUUACUUAUUUAUUCGCCC	171
7C-22: (1)	UGCUCUUAAGGAACCAUCGUCUAUAUCCCAUUCUGACUGCC	172
7C-30: (1)	UGCUCU CAGGAACCAUCGUUUUCCCAUGUCCUUCUGCC	173
7C-29: (3)	UGCUCUUGGAUUACCAAGGAACCAUUUCCUCUACCCCC	174

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<sup>a</sup>N7 fixed sequences are not shown. (5'-GGGAGGACGAUGCGG-N-CAGACGACUCGCCCCGA-3')

**Table 10.** 30N8 sequences isolated from a plate SELEX on human c-met.

Clone name: (number of isolates).      Sequence<sup>a</sup>      SEQ ID NO:

**FAMILY 1:**

7b-1:	(4)	GUGCUCAUUACGAACUUGACCGAUGCCUA	175
7b-9:	(1)	GGUGCUCAUUACGAACUUGACCGAAGCCUA	176
7b-18:	(1)	GGUGCUCAUUACGAACUUGACCGAUGCCUA	177
7b-3:	(1)	AGUGCUCCAAUGAACUUUGCUCGCUGA	178
7b-8:	(1)	GGUGCUCCGUUUGGAACUUGAUCGGUAGGA	179
7b-7:	(1)	GUGCUCAUUCAGAACUUGACGUUAACCA	180
7b-14:	(1)	GGUGCUCUUAGGAACUUGACCGUCCGCCA	181
7b-16:	(1)	GUGGUGCUCACUAACCAAGUGGAACCUUG	182
consensus:		GUGCUC-UU--GAACUUGACCG	183

**OTHERS:**

7b-10:	(1)	ACGAUAAGUGGGAGUGAGUAAGUUUGAGUA	184
7b-12:	(1)	CCUAGACCCCCAGGUUCCUCCCCACUAGUC	185

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<sup>a</sup>N8 fixed sequences are not shown. (5'-GGGAGAUAAAGAAUAAACGCUCAA-N-UUCGACAGGAGGCUCACAACAGGC-3')

**Table 11.**  $\alpha_v\beta_3$  Family 1 aptamer sequences.

Clone name (# of isolates)	Sequence of variable region 5'-gggagacaagaauaacgcuaa [40N] uucgacaggaggcucacaacaggc-3'	Seq. length	K <sub>D</sub> (nM)	SEQ ID NO: 310
7.3 (2)	Uucuaacgu uguuuuaggggcuuauaugagcgcauuauaccc	40	22	194
7.6; 17.12A	Uucaacgc uguuuuaggggcuuauaugagcgcuuauaccc	40	ND	195
7.12	Uucaacgc uguuuuaggggcuuauaugagcgcuuacaccc	40	ND	196
7.24 (5)	Uucaacgc uguucaaggggcuuauaugagcgcuuauaccc	40	170	197
7.25	Uucaacga uguuuuaggggcuuauaugagcgcuuauaccc	40	ND	198
7.34	uucau gaa guccaaggggcuuauaugagcgcuuauaccc	39	ND	199
7.36 (3)	Uucaacgc ugucaaaggggcuuauaugagcgcuuauaccc	40	ND	200
7.37 (2)	uu aacgu uguucaagggguuauaugagugcgcuuauaccc	39	ND	201
7.38 (2)	Uucaacgc uguccaaggggcuuauaugagcgcuuauaccc	40	49	202
7.49	Uucaacggauguccaagggcuu uaugagcgcuuauaccc	40	ND	203
7.53	Uucgacgc uguucaaggggcuuauaugagcgcauuauaucc	40	ND	204
7.54 (2)	Uucgacgc uguucaaggggcuuauaugagcgcuuauaccc	40	230	205
7.57 (2)	Uucgacga uguccaaggggcuuauaugagcgcauuauaccc	40	ND	206
7.63	Uucaacgc uguucaaggggcuuauaugagcgcuuacaccc	40	ND	207
7.64	uuc auga uguucaaggggcuuauaugagcgcauuauaccc	39	ND	208
7.77 (2)	Uucaacga uguugaggggcuuauaugagcgcauuauaccc	40	770	209
7.80	Uucaacga uguccaaggggcuuauaugagcgcauuauaccc	40	ND	210
7.86	Uucaacgc uguucaaggggcuuauugagcgcuuauaccc	40	ND	211
7.91	Uucaacgu uguccaaggggcuuauaugagcgcauuauaccc	40	ND	212
7.115	Uucaacgc uguucaaggggcuuauaugagcgcauuauaccc	40	ND	213
7.121	Uucaacga uguccaaggggcuuauaugagcggaaua ccc	38	ND	214
7.124	Uucaacac ugu gaagggcuuauaugagcgcuuauaccc	39	ND	215
7.127	Uucaacgu uguucaaggggcuuauaugagcgcuuauaccc	40	ND	216
15.2	Uucaacgu ugucaaaggggcuuauaugagcggaaua ccc	38	6	217
15.3 (3); 17.17	Uucaacgu uguccaaggggcuuauaugagcggaaua ccc	38	8	218
15.7	Uucuaacga ugucaaaggggcuuauaugagcggaaua ccc	38	5	219
15.8	Uucgacgc uguugaaggggcuuauacgagcggaaua ccc	38	5	220
15.10	Uucaacgc uguucaaggggcuuauaugagcggaaua ccc	38	20	221
15.14	Uucaacau uguccaaggggcuuauaugagcggaaua ccc	38	6	222
15.17	Uucaacgu ugucaaaggggcuuauacggcggaaua ccc	38	4	223

Table 11. (Cont.)

Clone name (# of isolates)	Sequence of variable region 5'-gggagacaagaauaaacgcuaa [40N] uucgacaggaggcucacaacaggc-3'			Seq. length	K <sub>D</sub> (nM)	SEQ ID NO: 310	
15.18 (2)	Uucaacgc	ugug	aagggcuaauaugagcggauua	ccc	37	2	224
15.20	Uucaacgc	uguccaagggcuaauaugagcgcauuauaccc		40	20	225	
15.27	Uucgacua	uguccaagggcuaauaugagcggauua	ccc	38	ND	226	
15.28	Uucgacga	ugucuaagggcuaauaugagcggauua	ccc	38	ND	227	
15.40; 17.12B	Uucaacgc	uguugaagggcuaauacgagcggauua	ccc	38	ND	228	
15.41	Uucaacgu	uguccaagggcuaauacgagcggauua	ccc	38	ND	229	
15.42; 17.14	Uucaacgc	uguccaagggcuaauacgagcggauua	ccc	38	ND	230	
(2)							
15.46; 17.20	Uucgacgc	ugug	aagggcuaauaugagcggauua	ccc	37	40	231
15.47	Uucaacgu	ugucuaagggcuaauacgagcggauua	ccc	38	ND	232	
15.48	Uucaacgc	uguugaagggcuaauaugagcggauua	ccc	38	ND	233	
15.49	Uucuaacgu	ugucuaagggcuaauaugagcggauua	ccc	38	ND	234	
15.50; 17.3	Uucgacgc	ugug	aagggcuaauacgagcggauua	ccc	37	30	235
15.52	Uucaacgc	uguucaagggcuaauacgagcggauua	ccc	38	ND	236	
15.53	Uucaacgc	uguccuagggcuaauaugagcgaggauaccc		40	70	237	
15.55	Uucuaacgc	uguuuuagggcuaauaugagcgaaaua	ccc	38	ND	238	
15.57	Uucuaacgu	uguccaagggcuaauaugagcggauua	ccc	38	ND	239	
15.58	Uucgacgu	uguugaagggcuaauaugagcggauua	ccc	38	ND	240	
17.1	Uucaacgc	ugucuaagggcuaauauaagcggauua	ccc	38	380	241	
17.2 (2)	Uucuaacgc	ugug	aagggcuaauaugagcggauua	ccc	37	2	242
17.5	Uucgacgc	ugug	aagggcuaauaugagcggau acaccc	38	5	243	
17.7 (2)	Uucuaacgc	ugug	aagggcuaauacgagcggauua	ccc	37	6	244
17.8	Uucaacgu	ugucuaagggcuaauaugagcggauua	ccc	38	18	245	
17.10	Uucuaacgu	uguugaagggcuaauaugagcggauua	ccc	38	ND	246	
17.11	Uucuaacgc	ugug	aagggcuaauaugagcgaaaua	ccc	37	4	247
17.13	Uucaacgc	uguccaagggcuaauaugggcggauua	ccc	38	10	248	
17.16	Uucaacgc	ugug	aagggcuaauacgagcggauua	ccc	37	8	249

Table 12.  $\alpha_v\beta_3$  Family 2 aptamer sequences

Clone name (# of isolates)	Sequence of variable region 5'-gggagacaagaauaaacgcucuaa [40N] uucgacaggaggcucacacag gc-3'	Seq. length	K <sub>D</sub> (nM)	SEQ ID NO: 310
7.4	GUACCGGAUCGCCCUGCCACGGUAUUUGAGACAUUGAAA	39	ND	250
7.5 (3)	GGUAGUAAAUGGACUCCUGCCAUCCAAUACUAUCUCUGAG	40	>1000	251
7.13	UGUAGUCGCAUGUCGAGCAGCAAUUCUGCCAUGUAGG	39	>1000	252
7.14 (2)	UGAAGAACUAGACCUGCCCAAGUCCUUAUCGUGCUUGCU	40	ND	253
7.27 (2)	CGAUUAUACUAUCCCUGCCAGUAGUAAUCAGUGCUAUA	38	ND	254
7.29	CGGUGAAGACCUCUAUUAACAACAUAGACCUGCCUGCGUUG	40	ND	255
7.32	CGCAAAUAUGUUCUGCCAAAUACGGGCGUUGACGCUAGA	40	ND	256
7.43	GGACCCUGCCGAGCACAUAUUUAUUCUGGUAACUGAGCCCCC	40	ND	257
7.51	CGCUGAGAGAAAGCCUGCCCUUUCAGCUCGAGAGUUAUA	40	ND	258
7.58	UGAGAUGCAGUUCUGCCUGCUGCAUUUCUAGAGUGUGU	40	ND	259
7.83	GAUUAACGGUUAUCCUGCCAACCGAUUAUAAGAGCAUGGA	40	ND	260
7.89	UGAGAGACUACAAUAGAACUUAUGUAACCUGCCACAUAGG	40	ND	261
7.97	UAGGAAGUGUAACCUGCCUCACGGUCCUAUCGAGUAGUUU	40	ND	262
7.100	UGAAAACGCAACCUGCCGGCGUCGUCCUUUGGGUAAUUUA	40	ND	263
7.104	AUAGGGGGUUAACCUGCCGACCCAGAAUAAGCGUGAUU	39	ND	264
7.105	UCCUGCCAUAAGCGUCUUAUGUCUGACGUUUGAGUUUCCG	40	ND	265
7.107	UCCUAGGUUGGUCCUGCCACAGCUCAAAGGUUUAAGCUUCA	40	ND	266
7.109	ACAUGCAGACAACCCUGCCUUCUGCGUGGUUUAAGGAGUA	39	ND	267
7.120	AACCUCAGGCGACCUGCCGCGUCUGAGUUCGAGCAUAA	40	ND	268
7.122	ACUCAAGA'CCCUGCCACUAUGUGUUAUCUGAGUAGGAGCGU	40	ND	269
7.125	AUUCGAAAUACGGGUUAAAUCCUGCCUUAACACGACA	39	ND	270
15.19	UGUAGCCGCAUGUCGAGCAGCAAUUCUGCCAUGUAGG	39	770	271
15.21	CGGUGAAGACCUCUAUUAACAACAUGACCUGCCUGCGUUG	40	200	272
15.34	UCCCACCCUGCCUUGUCUGUUUGAUAGAGACACUGUCCUU	40	190	273

**Table 13.**  $\alpha_v\beta_3$  orphan aptamer sequences

Clone name (# of isolates)	Sequence of variable region 5'-gggagacaagaauaacgcucuaa [40N] uucgacaggaggcucacacaggc-3'	Seq. length	K <sub>D</sub> (nM)	SEQ ID NO: 310
7.1	gguuugaaagauugccuguagcuccaaaucuuuggugagcu	40	ND	274
7.2	ucccgccgauagcuuccacgaagaguuaucuguaaaacaa	40	ND	275
7.11	ugagcuccugauuccaaaaccuauuccguuucugggu	36	ND	276
7.30	acuggacaagucaaucucuccggcuugagacuugguuuac	40	ND	277
7.33 (2)	cgagcucuugcuuccaaaaccuauuccagacguuu cuggg	40	ND	278
7.41 (2)	gcgagccuauugucuaagaugcaccaggccuguaaagcau	40	>1000	279
7.42	gccuguacggcgauuaugucuuuaccuuacugucc	37	ND	280
7.46	uaccaauggcacgaauaacugacuaccccccaaauggaa	40	ND	281
7.47	gcggggcuuugcucaaguguuugcaaacgguaaaauccac	40	ND	282
7.61	ccuaccgacgucggcgccguggguaaacuguaaagucacu	40	ND	283
7.66 (2)	gugaaccgaaagcgaaaguacccccugcuugacuacu	40	>1000	284
7.67	ggagcuccuaguuccaaaaccuauuccagaaguuuucugggu	41	ND	285
7.75	uaguacgcagucauagcggggcgaggacuucuccgugca	40	ND	286
7.76	uuauacugguauugccgcccagcagaauuaauccauugcu	40	ND	287
7.82	ugagcuccugguuccaaaaccuauuccagacguuucagggu	40	ND	288
7.85	ucuggccugugacugugucguuucucgaguugugacgc	40	ND	289
7.92	cuaacgauguccaaggccuauauagagcgcguaacccc	39	ND	290
7.93	gcgagccuauugucuaagaugcgccaagccuguaaagcau	40	ND	291
7.94	gacuagccggccugagauccuuguucgccacacaugcugg	40	ND	292
7.96	cuccccccgcaaacacauguuuaguacugggagacuuggg	40	ND	293
7.101	ugagcuccugauuccgaaccuauuccagacguuucugggu	40	ND	294
7.102	cugauccucuugucauuguacaucucgcag	30	ND	295
7.106	uacuaagccuaacaaaagagcggaauuuggcgcgccacg	39	ND	296
7.108	agucuuaguaguaccgcccugcuucuaaccuugggcgcuuu	40	ND	297
7.112	ugauuucaugacuuaugccgcccggcaugacuucacugacg	40	ND	298
7.114	ucaaaggacggaagugccugugcccgcuaaaagaguugag	40	ND	299
7.118	Cuaucgaucguuuuucauuuucccccugaccaucgccug	39	ND	300
7.123	Uugucccgcgagaaacgugacaaauuaacacgcaccgu	40	ND	301
7.128	Uucaacguuguucaaggccuauauagagcgcguaauaccc	40	ND	302
15.4 (4)	Ugauuucaugacuuaugccgcccggcaugacuucacugacg	40	2000	303
15.5	Gcauucaaaauuugcgagaacgaauagaaguccgagagcc	40	4000	304
15.13	Gcgggaauuuuccugaucauuccacugauucggggccuac	40	790	305
(2)				
15.39	Ucaaucucggacuagacuacgaccuugguugacgcuca	39	410	306
15.43	Cgcccguauacacgacgugcguucuggggcgguacucgcgca	40	45	307

What is claimed is:

1. A nucleic acid ligand to hepatocyte growth factor/scatter factor (HGF) identified according to the method comprising:

- a) preparing a candidate mixture of nucleic acids;
- 5        b) contacting the candidate mixture of nucleic acids with HGF, wherein nucleic acids having an increased affinity to HGF relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;
- c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and
- 10       d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acids with relatively higher affinity and specificity for binding to HGF, whereby a nucleic acid ligand of HGF may be identified.
2. A purified and isolated non-naturally occurring nucleic acid ligand to HGF.
3. A purified and non-naturally occurring RNA ligand to HGF wherein said ligand  
15 is selected from the group consisting of SEQ ID NOS:12-14 in Figure 7, SEQ ID NOS:15-17 in Figure 8, SEQ ID NOS:18-93 in Table 2, SEQ ID NOS:94-131 in Table 3, SEQ ID NOS:132-155 in Table 5, and SEQ ID NOS:156-159 in Table 7.
4. The nucleic acid ligand of claim 1 wherein HGF is associated with a solid support, and wherein steps b)-c) take place on the surface of said solid support.
- 20       5. The nucleic acid ligand of claim 4 wherein said solid support is comprised of nitrocellulose.
6. The nucleic acid ligand of claim 1 wherein said candidate mixture of nucleic acids is comprised of single stranded nucleic acids.
7. The nucleic acid ligand of claim 6 wherein said single stranded nucleic acids are  
25 ribonucleic acids.
8. The nucleic acid ligand of claim 6 wherein said single stranded nucleic acids are deoxyribonucleic acids.
9. The nucleic acid ligand of claim 7 wherein said candidate mixture of nucleic acids comprises 2'-F (2'-fluoro) modified ribonucleic acids.
- 30       10. The purified and isolated non-naturally occurring nucleic acid ligand of claim 2 wherein said nucleic acid ligand is single stranded.



11. The purified and isolated non-naturally occurring nucleic acid ligand of claim 10 wherein said nucleic acid ligand is RNA.

12. The purified and isolated non-naturally occurring RNA ligand of claim 11 wherein said ligand is comprised of 2'-fluoro (2'-F) modified nucleotides.

5 13. A method for the isolation of nucleic acid ligands to HGF, comprising:

a) preparing a candidate mixture of nucleic acids;

b) contacting the candidate mixture of nucleic acids with HGF, wherein nucleic acids having an increased affinity to HGF relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

10 c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acids with relatively higher affinity and specificity for binding to HGF, whereby a nucleic acid ligand of HGF may be identified.

15 14. The method of claim 13 wherein said candidate mixture comprises single-stranded nucleic acids.

15. The method of claim 14 wherein said single-stranded nucleic acids comprise ribonucleic acids.

20 16. A method for the treatment of a tumor comprising administering a biologically effective dose of a nucleic acid ligand to HGF.

17. A method for determining the level of HGF in an individual comprising:

a) providing a nucleic acid ligand to HGF;

b) contacting a biological fluid from said individual with said nucleic acid ligand; and

25 c) determining the amount of HGF that has bound to said nucleic acid ligand.

18. A method for inhibiting angiogenesis, the method comprising administering a biologically-effective dose of a nucleic acid ligand to HGF.

19. A pharmaceutical composition for the treatment of a tumor comprising a nucleic acid ligand to HGF and a pharmaceutically acceptable excipient.

30 20. A nucleic acid ligand to c-met identified according to the method comprising:

a) preparing a candidate mixture of nucleic acids;

b) contacting the candidate mixture of nucleic acids with c-met, wherein nucleic acids having an increased affinity to c-met relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

5 c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acids with relatively higher affinity and specificity for binding to c-met, whereby a nucleic acid ligand of c-met may be identified.

21. A purified and isolated non-naturally occurring nucleic acid ligand to c-met.

10 22. A purified and non-naturally occurring RNA ligand to HGF wherein said ligand is selected from the group consisting of SEQ ID NOS:160-174 in Table 9 and SEQ ID NOS:175-185 in Table 10.

23. The nucleic acid ligand of claim 20 wherein c-met is associated with a solid support, and wherein steps b)-c) take place on the surface of said solid support.

15 24. The nucleic acid ligand of claim 23 wherein said solid support is comprised of nitrocellulose.

25. The nucleic acid ligand of claim 20 wherein said candidate mixture of nucleic acids is comprised of single stranded nucleic acids.

20 26. The nucleic acid ligand of claim 25 wherein said single stranded nucleic acids are ribonucleic acids.

27. The nucleic acid ligand of claim 25 wherein said single stranded nucleic acids are deoxyribonucleic acids.

28. The nucleic acid ligand of claim 26 wherein said candidate mixture of nucleic acids comprises 2'-F (2'-fluoro) modified ribonucleic acids.

25 29. The purified and isolated non-naturally occurring nucleic acid ligand of claim 21 wherein said nucleic acid ligand is single stranded.

30. The purified and isolated non-naturally occurring nucleic acid ligand of claim 29 wherein said nucleic acid ligand is RNA.

30 31. The purified and isolated non-naturally occurring RNA ligand of claim 30 wherein said ligand is comprised of 2'-fluoro (2'-F) modified nucleotides.

32. A method for the isolation of nucleic acid ligands to c-met, comprising:

- a) preparing a candidate mixture of nucleic acids;
  - b) contacting the candidate mixture of nucleic acids with c-met, wherein nucleic acids having an increased affinity to c-met relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;
  - 5 c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and
  - d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acids with relatively higher affinity and specificity for binding to c-met, whereby a nucleic acid ligand of c-met may be identified.
- 10 33. The method of claim 32 wherein said candidate mixture comprises single-stranded nucleic acids.
34. The method of claim 33 wherein said single-stranded nucleic acids comprise ribonucleic acids.
35. A method for the treatment of a tumor comprising administering a biologically  
15 effective dose of a nucleic acid ligand to c-met.
36. A method for inhibiting angiogenesis, the method comprising administering a biologically-effective dose of a nucleic acid ligand to c-met.
37. A pharmaceutical composition for the treatment of a tumor comprising a nucleic acid ligand to c-met and a pharmaceutically acceptable excipient.
- 20 38. A method for treating a disease in which elevated HGF is a causative factor, the method comprising administering a biologically-effective dose of a nucleic acid ligand to HGF.
39. A method for inhibiting tumor development, the method comprising administering a biologically effective dose of a nucleic acid ligand to HGF in combination  
25 with a biologically effective dose of a nucleic acid ligand to vascular endothelial growth factor (VEGF).
40. A method for inhibiting tumor development, the method comprising administering a biologically effective dose of a nucleic acid ligand to HGF in combination with a biologically effective dose of a nucleic acid ligand to basic fibroblast growth factor  
30 (bFGF).

41. A method for inhibiting tumor development, the method comprising administering a biologically effective dose of a nucleic acid ligand to HGF in combination with a biologically effective dose of a nucleic acid ligand to vascular endothelial growth factor (VEGF) and a biologically effective dose of a nucleic acid ligand to basic fibroblast growth factor (bFGF).

42. A method for inhibiting tumor development, the method comprising administering biologically effective doses of nucleic acid ligands to at least two growth factors.

43. The method of claim 42 wherein said growth factors are selected from the group consisting of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF $\beta$ ), HGF, and keratinocyte growth factor (KGF).

44. A method for inhibiting tumor development, the method comprising administering biologically effective doses of nucleic acid ligands to at least two receptors of growth factors.

45. The method of claim 44 wherein said growth factors are selected from the group consisting of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF $\beta$ ), HGF, and keratinocyte growth factor (KGF).

46. A method of inhibiting tumor development, the method comprising administering biologically-effective doses of nucleic acid ligands to one or more receptors of growth factors in combination with biologically-effective doses of nucleic acid ligands to one or more growth factors.

47. The method of claim 46 wherein said growth factors are selected from the group consisting of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF $\beta$ ), HGF, and keratinocyte growth factor (KGF).

48. A nucleic acid ligand to an integrin identified according to the method comprising:

a) preparing a candidate mixture of nucleic acids;

b) contacting the candidate mixture of nucleic acids with said integrin, wherein nucleic acids having an increased affinity to said integrin relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

5 c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acids with relatively higher affinity and specificity for binding to said integrin, whereby a nucleic acid ligand of said integrin may be identified.

49. The nucleic acid ligand of claim 48 wherein said integrin is a  $\beta_3$  integrin.

10 50. The nucleic acid ligand of claim 49 wherein said  $\beta_3$  integrin is selected from the group consisting of  $\alpha_{\text{mb}}\beta_3$  integrin and  $\alpha_v\beta_3$  integrin.

51. A purified and isolated non-naturally occurring nucleic acid ligand to an integrin.

15 52. A purified and non-naturally occurring RNA ligand to an integrin wherein said ligand is selected from the group consisting of SEQ ID NO:194-307.

53. The nucleic acid ligand of claim 48 wherein said integrin is associated with a solid support, and wherein steps b)-c) take place on the surface of said solid support.

54. The nucleic acid ligand of claim 53 wherein said solid support is a bead

20 55. The nucleic acid ligand of claim 48 wherein said candidate mixture of nucleic acids is comprised of single stranded nucleic acids.

56. The nucleic acid ligand of claim 55 wherein said single stranded nucleic acids are ribonucleic acids.

57. The nucleic acid ligand of claim 55 wherein said single stranded nucleic acids are deoxyribonucleic acids.

25 58. The nucleic acid ligand of claim 56 wherein said candidate mixture of nucleic acids comprises 2'-F (2'-fluoro) modified ribonucleic acids.

59. The purified and isolated non-naturally occurring nucleic acid ligand of claim 51 wherein said nucleic acid ligand is single stranded.

30 60. The purified and isolated non-naturally occurring nucleic acid ligand of claim 59 wherein said nucleic acid ligand is RNA.

61. The purified and isolated non-naturally occurring RNA ligand of claim 60 wherein said ligand is comprised of 2'-fluoro (2'-F) modified nucleotides.

62. A method for the isolation of nucleic acid ligands to an integrin, comprising:

a) preparing a candidate mixture of nucleic acids;

5 b) contacting the candidate mixture of nucleic acids with said integrin, wherein nucleic acids having an increased affinity to said integrin relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

10 d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched for nucleic acids with relatively higher affinity and specificity for binding to said integrin, whereby a nucleic acid ligand of said integrin may be identified.

63. The method of claim 62 wherein said candidate mixture comprises single-stranded nucleic acids.

15 64. The method of claim 63 wherein said single-stranded nucleic acids comprise ribonucleic acids.

65. The method of claim 62 wherein said integrin is a  $\beta_3$  integrin.

66. The method of claim 65 wherein said  $\beta_3$  integrin is  $\alpha_{IIb}\beta_3$  integrin.

67. A method for the treatment of a disease resulting from platelet activation, the  
20 method comprising administering a biologically-effective amount of a nucleic acid ligand to a  $\beta_3$  integrin.

68. A method for treating deep vein thrombosis comprising administering a biologically-effective amount of a nucleic acid ligand to a  $\beta_3$  integrin.

69. A pharmaceutical composition for the treatment of deep vein thrombosis  
25 comprising a nucleic acid ligand to a  $\beta_3$  integrin and a pharmaceutically acceptable excipient.

70. A method for detecting a deep vein thrombosis in an individual, the method comprising:

(a) providing a nucleic acid ligand to a  $\beta_3$  integrin, said nucleic acid ligand  
30 conjugated to a radioactive label;

(b) administering said nucleic acid ligand to said individual; and

(c) detecting the site of said thrombosis by analyzing the localization of said nucleic acid ligand using a radioimaging technique.

71. An anti-clotting composition for use in acute coronary syndromes and percutaneous coronary intervention, the composition comprising a nucleic acid ligand to a  
5  $\beta_3$  integrin and a pharmaceutically-acceptable excipient.

72. A method for the treatment of a disease in which  $\alpha_v\beta_3$  activation is a contributing pathogenic factor, the method comprising administering a biologically-effective dose of a nucleic acid ligand to  $\alpha_v\beta_3$  and a pharmaceutically acceptable excipient.

73. The method of claim 72 wherein said disease is selected from the group  
10 consisting of cancer, diabetic retinopathy, retinopathy of prematurity, macular degeneration, endometriosis, psoriasis, rheumatoid arthritis, stroke, osteoporosis, and restenosis.

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**N7 series**

Synthetic 307 or 40N7 DNA template: SEQ ID NO:1 (N = 30) and  
SEQ ID NO:15 (N = 40)

5'-TAATACGACTCACTATAGGGAUGACGAUGCGG-[N]<sub>30,40</sub>-CAGACGACUCGCCCCGA-3'

Starting random 30N7 or 40N7 RNA: SEQ ID NO:2 (N = 30) and  
SEQ ID NO:308 (N = 40)

5'-GGGAGGACGAUGCGG-[N]<sub>30,40</sub>-CAGACGACUCGCCCCGA-3'

**N7 primers**

5p7: 5'-TAATACGACTCACTATAGGGAUGACGAUGCGG-3' SEQ ID NO:3

3p7: 5'-TCGGGCGAGTCGTCTG-3' SEQ ID NO:4

**N8 series**

Synthetic 30N8 or 40N8 DNA template:

5'-TAATACGACTCACTATAGGGAGACAAGAATAAACGCTCAA-[N]<sub>30,40</sub>-  
TTCGACAGGAGGCTCACAACAGGC-3' SEQ ID NO:5

Starting random 30N8 or 40N8 RNA:

5'-GGGAGAUAAAGAAUAAACGCUCAA-[N]<sub>30,40</sub>-UUCGACAGGAGGCUCACAACAGGC-3'  
SEQ ID NO:6 (N = 30) and  
SEQ ID NO:309 (N = 40)

**N8 primers**

5p8: 5'-TAATACGACTCACTATAGGGAGACAA-3' SEQ ID NO:7

3p8: 5'-GCCTGTTGTGAGCCTCCTGTCGAA-3' SEQ ID NO:8

Fig. 1



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                                VV                V  
N7 RNA    5'-gggaggacgaugcgg-[N]-cagacgacucgcccga-3'  
            **cccuccugcuacgcc**-5'    gtctgcugagcgggcu-5'  
            SEQ ID NO:9                SEQ ID NO:10

Cleaved RNA            5'-(g)g-[N]-ca-3' SEQ ID NO:11

Fig. 2

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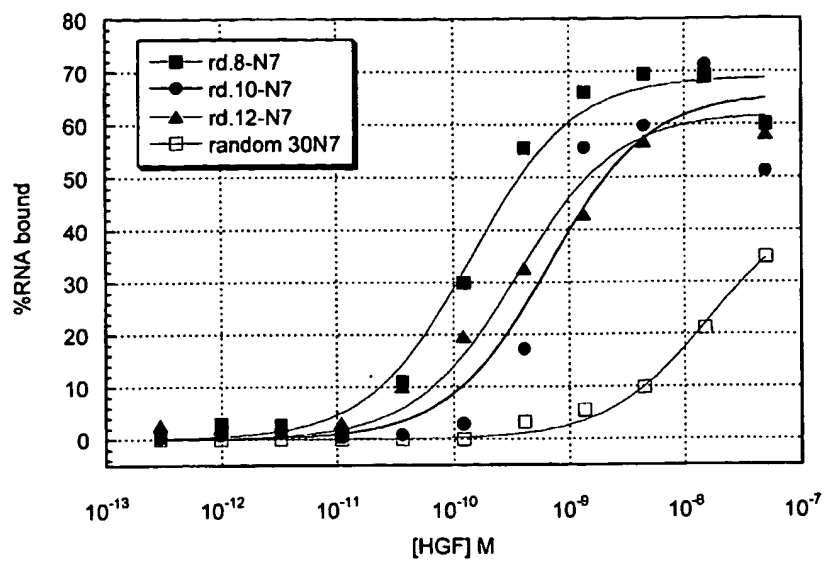


Fig. 3A

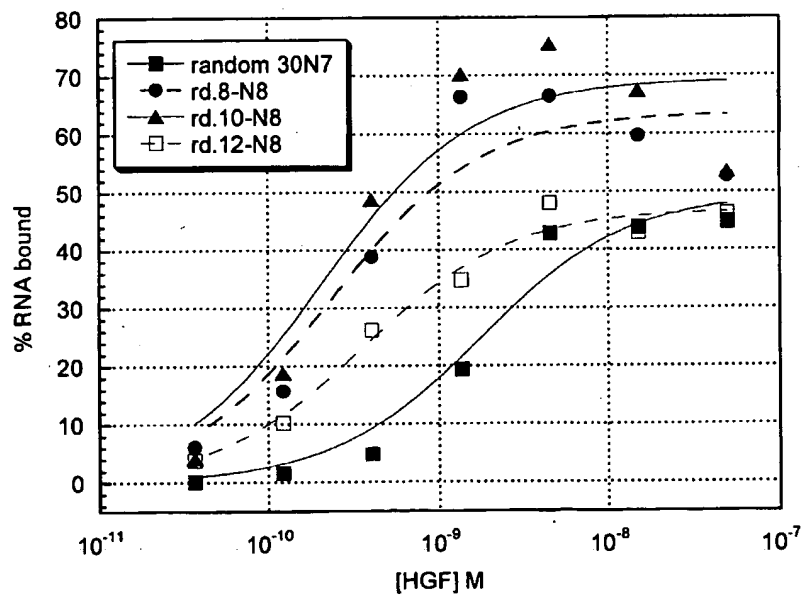


Fig. 3B

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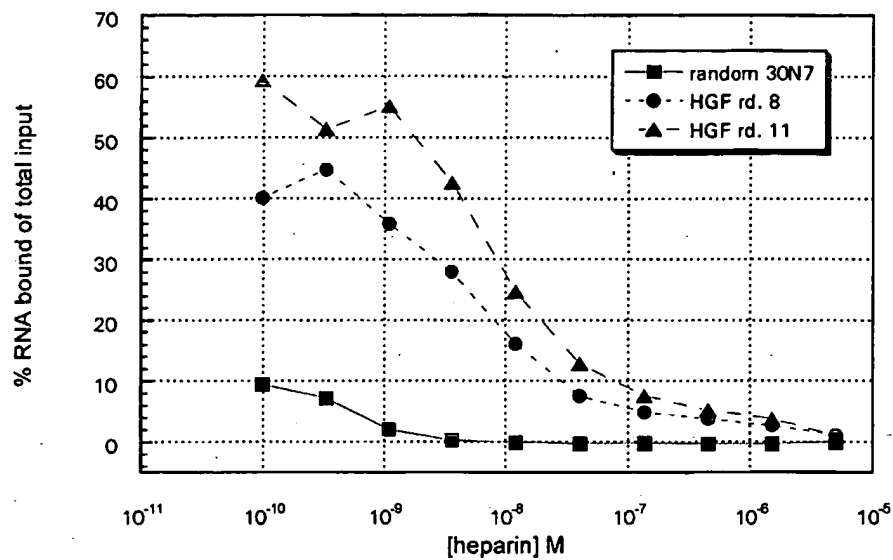


Fig. 4A

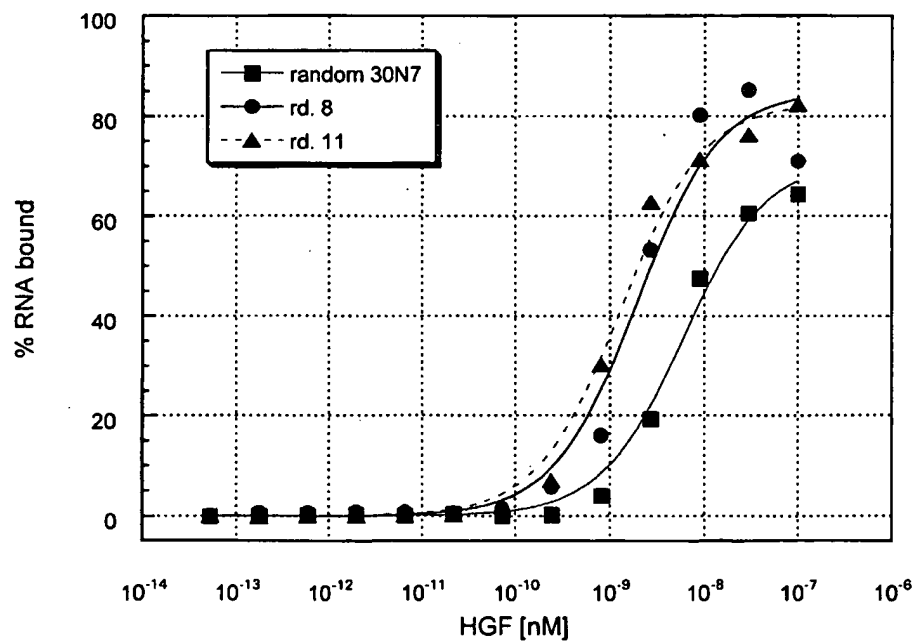


Fig. 4B

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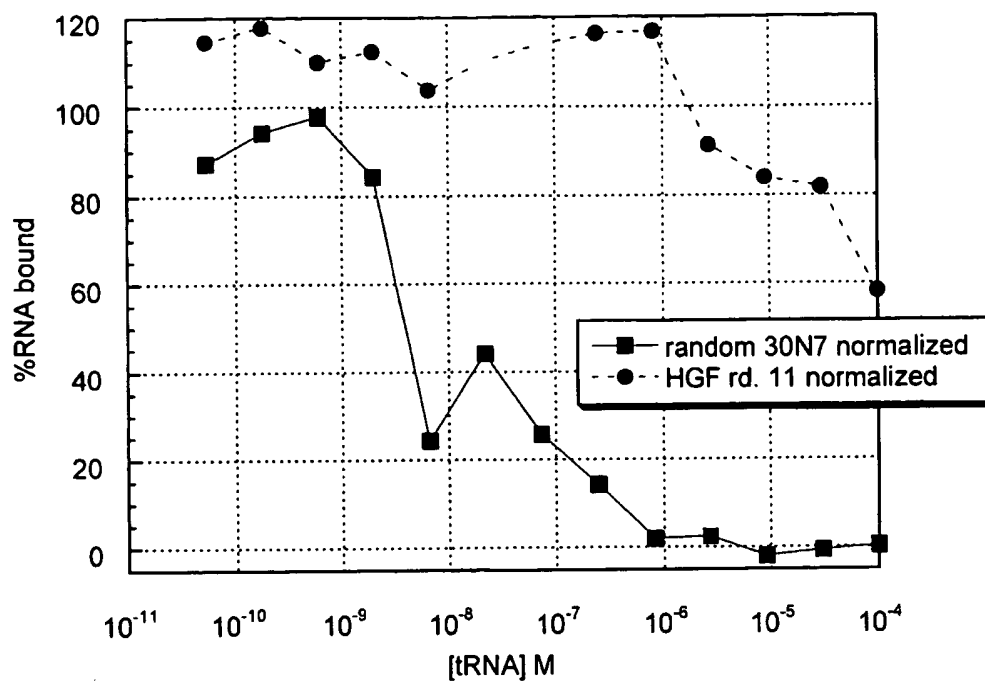


Fig. 5A

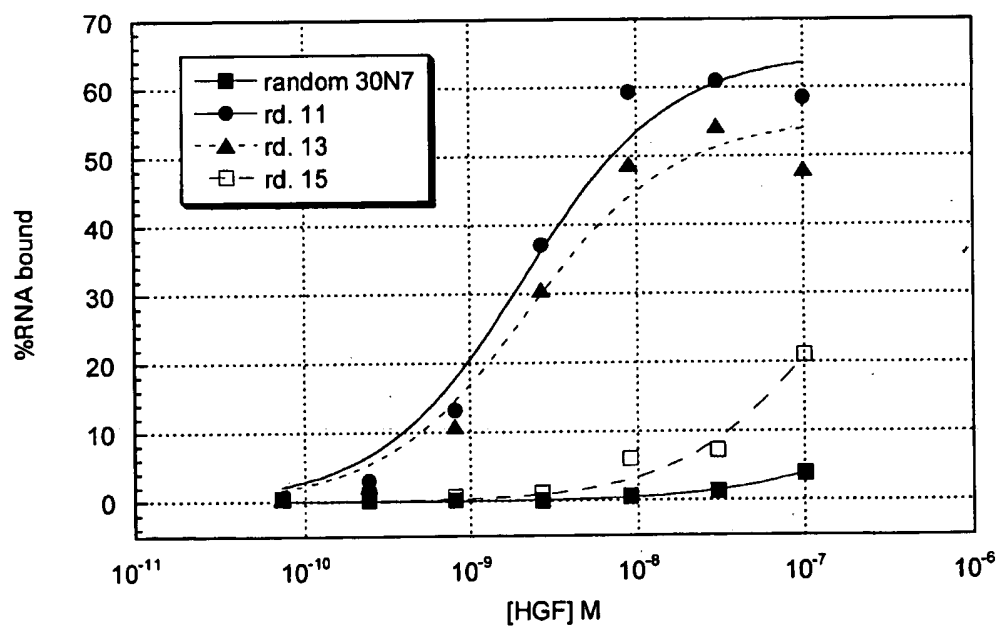


Fig. 5B

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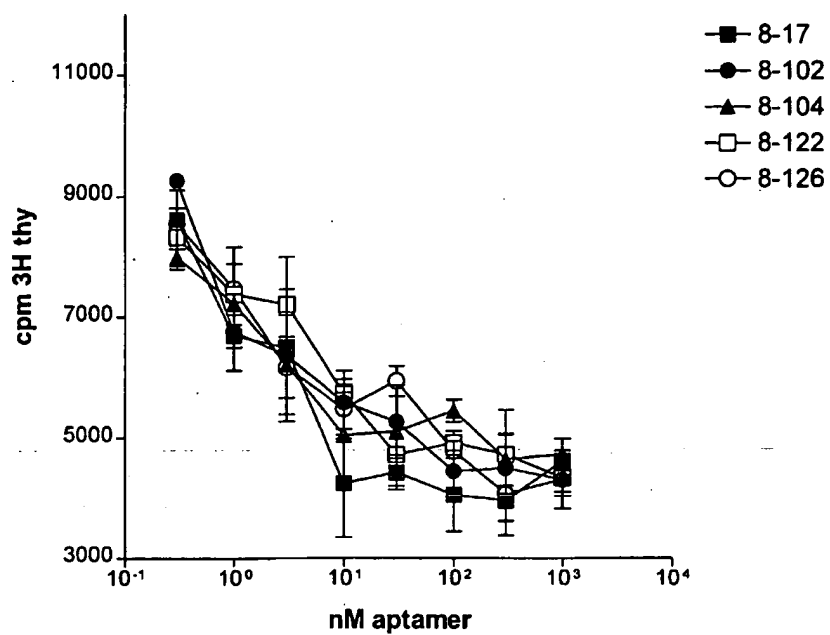


Fig. 6A

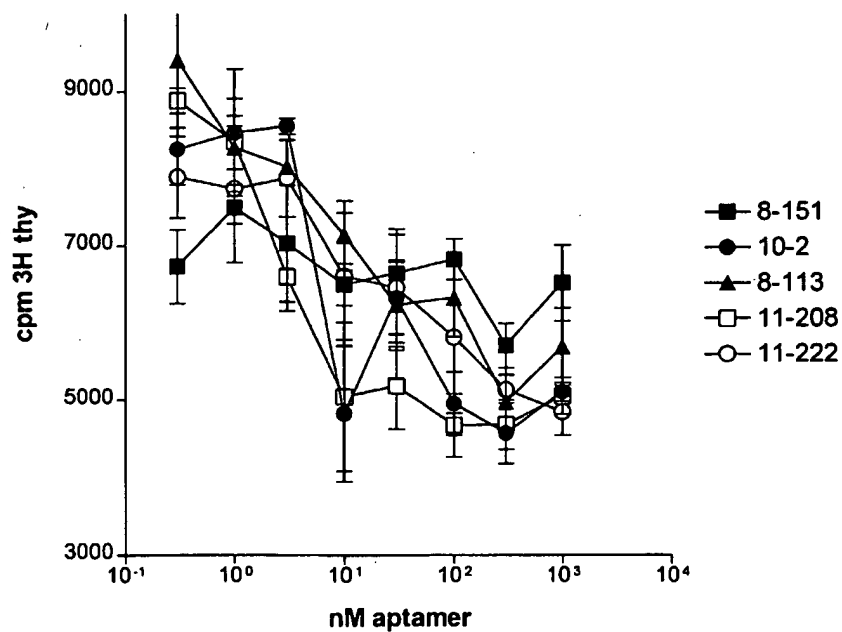


Fig. 6B

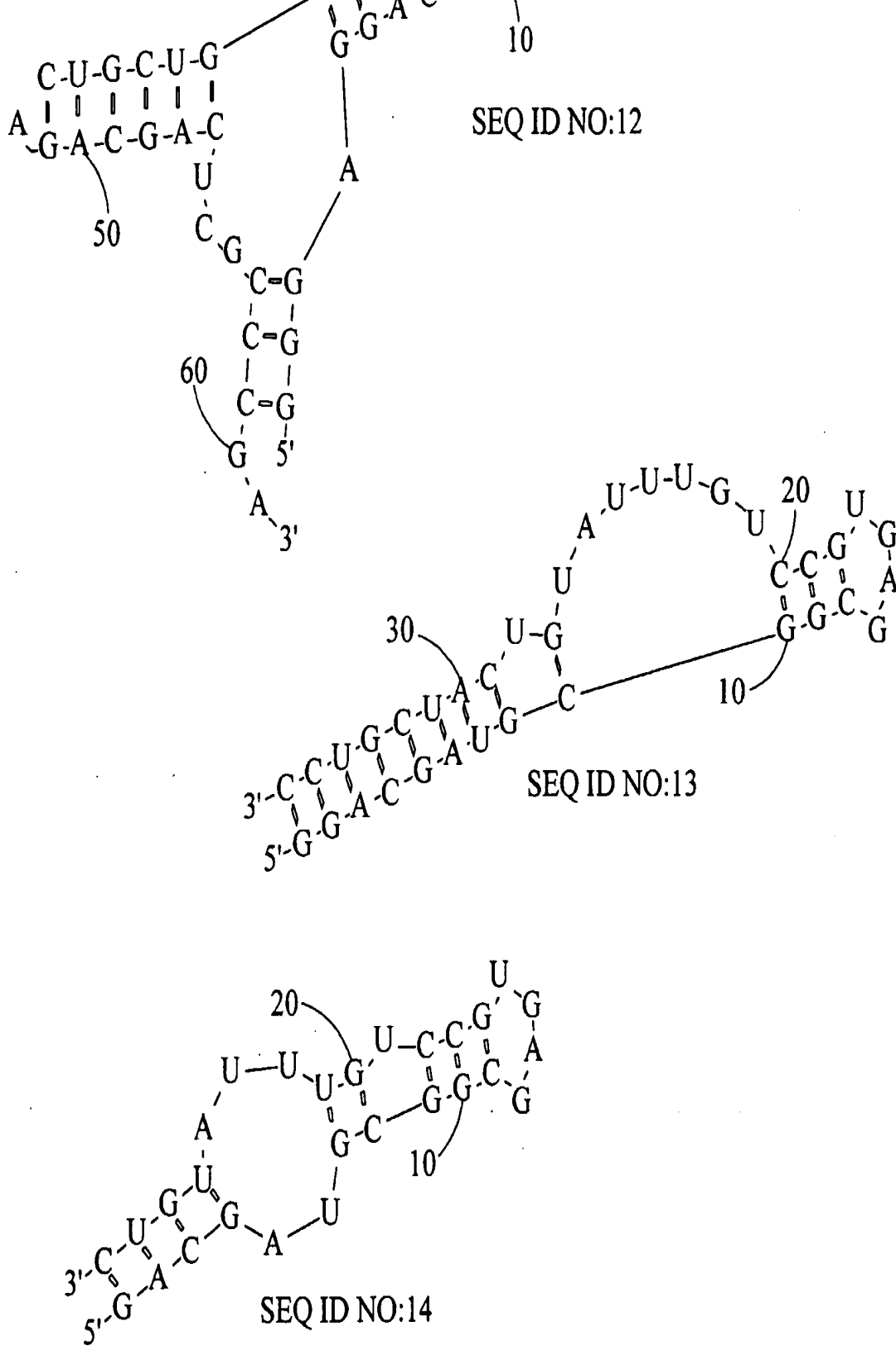


FIG. 7A

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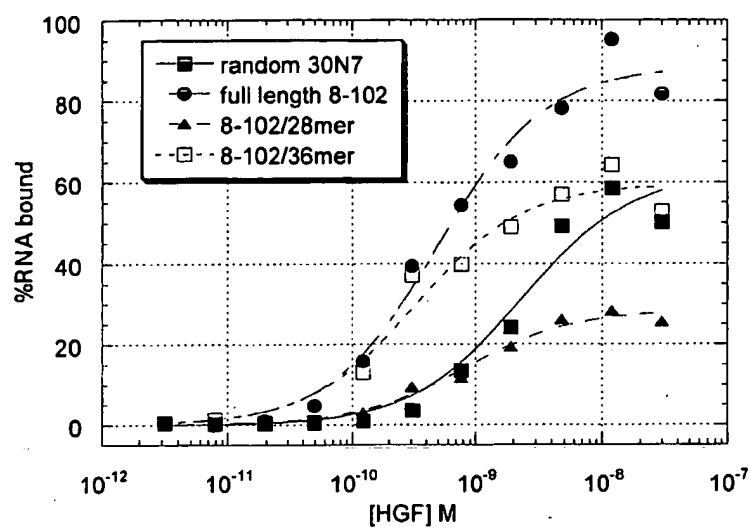


Fig. 7B

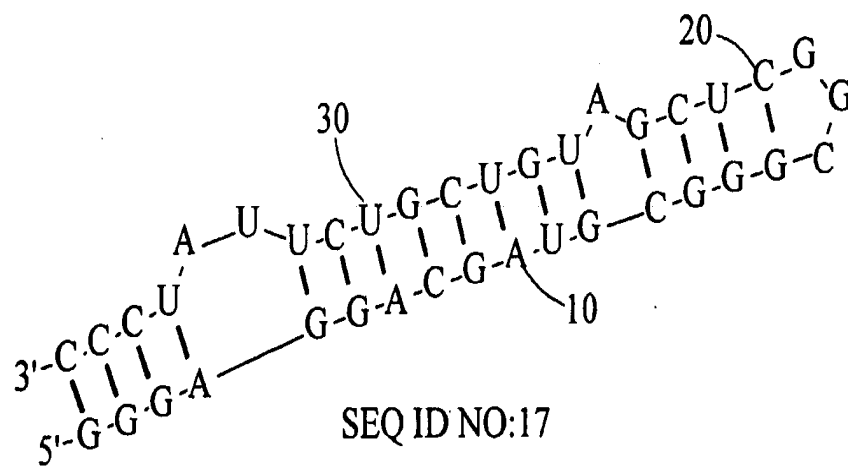
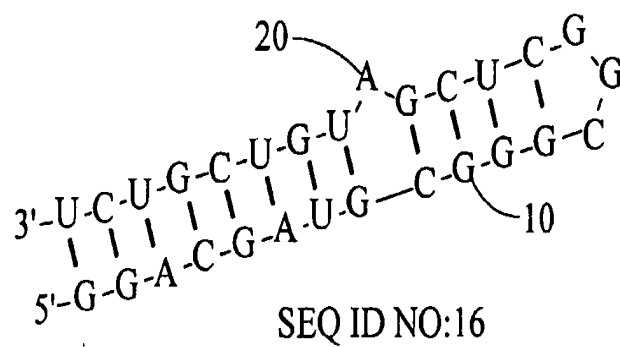
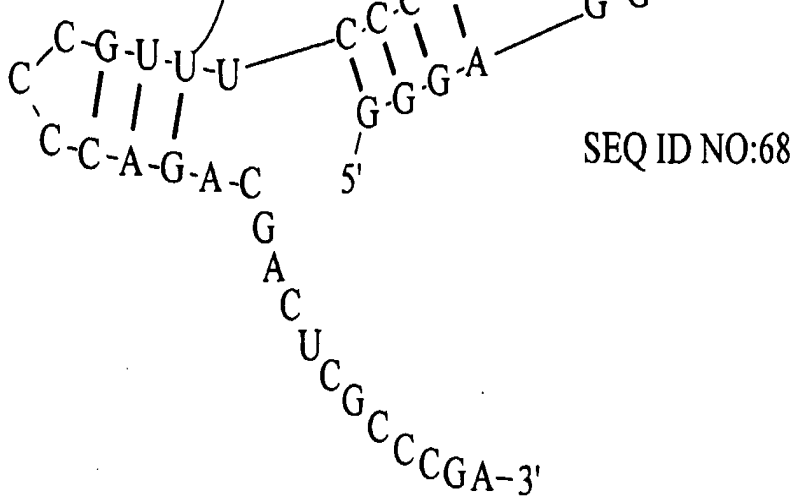


FIG. 8A



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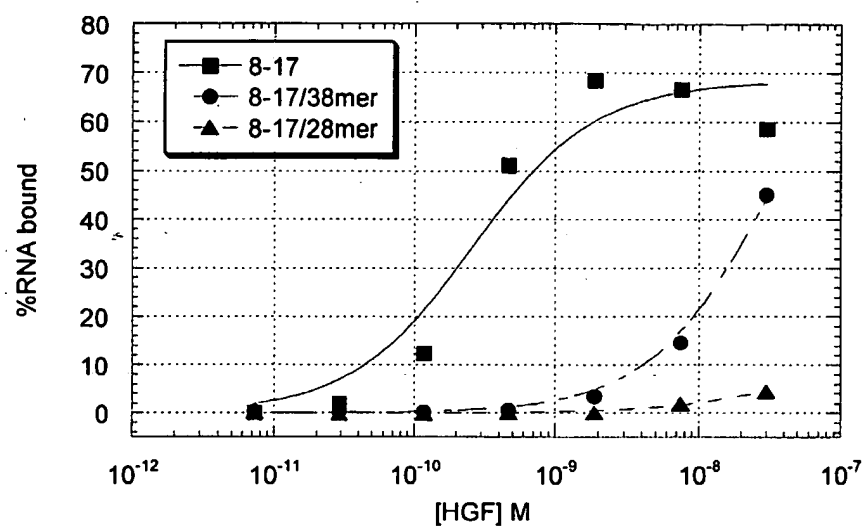


Fig. 8B

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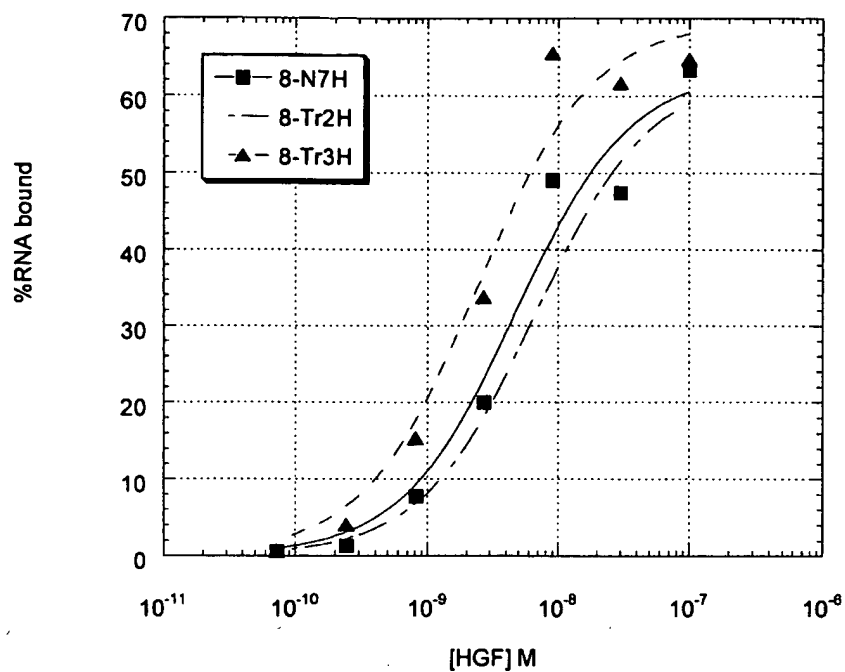


Fig. 9A

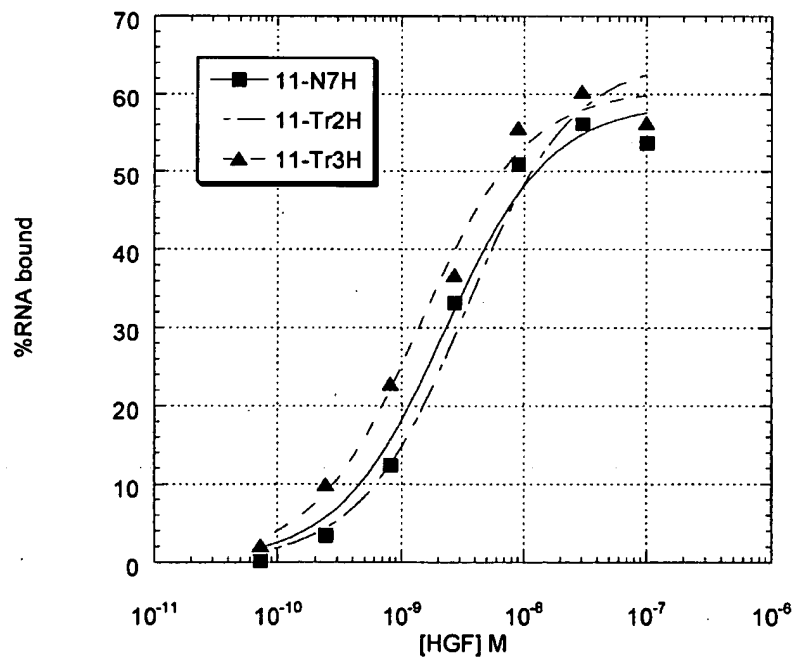


Fig. 9B

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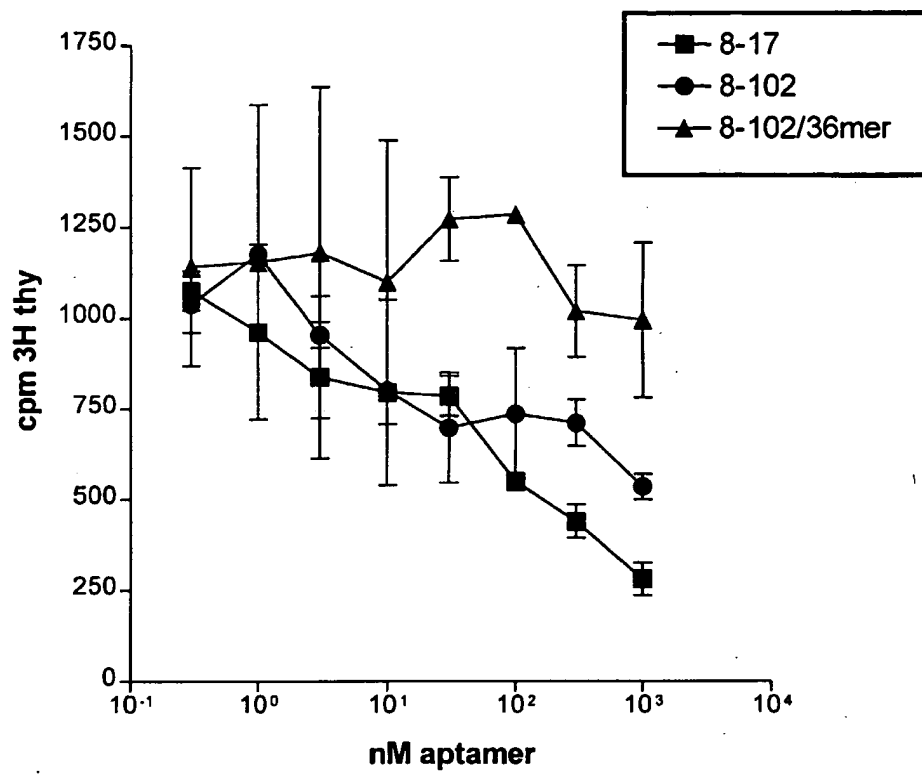


Fig. 10

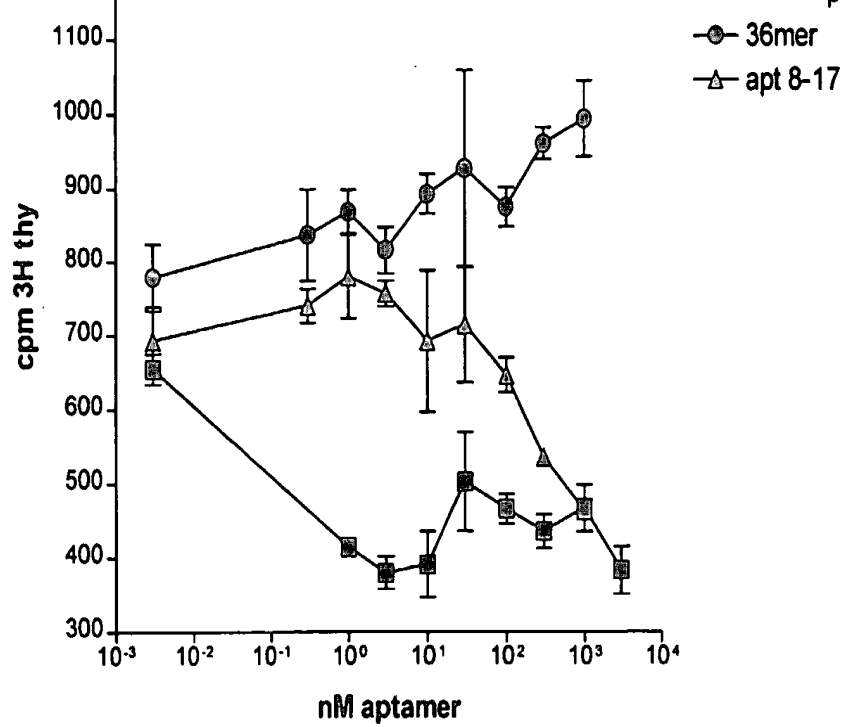


Fig. 11A

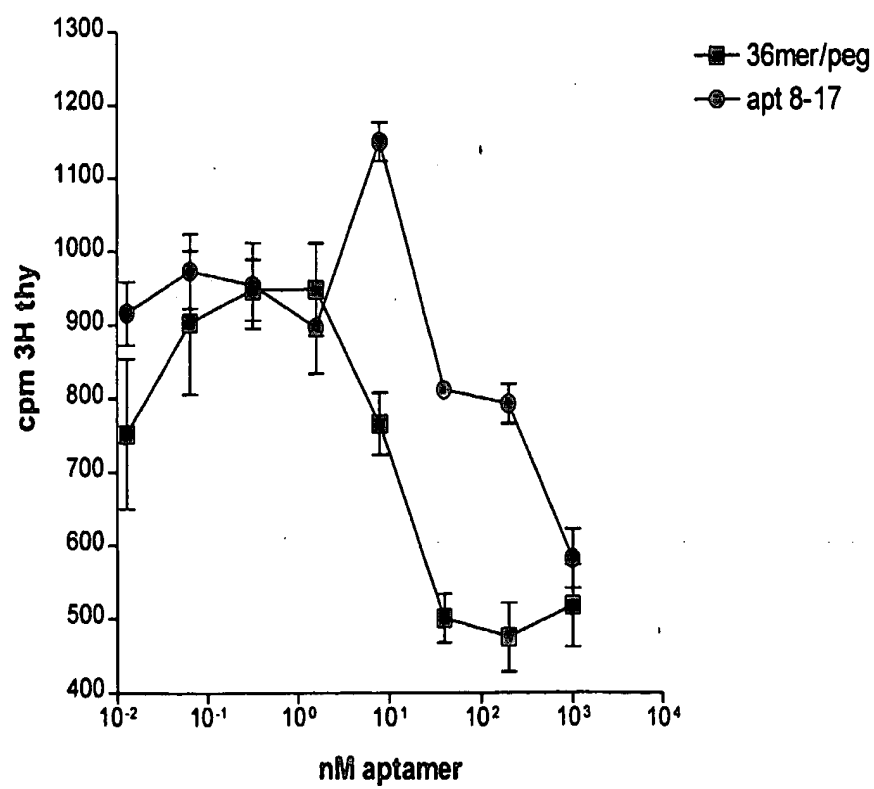


Fig. 11B

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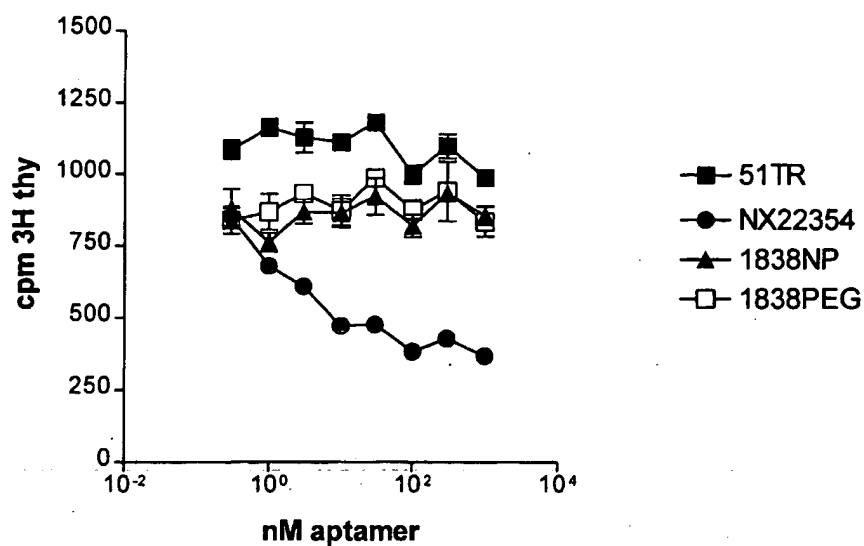


Fig. 12

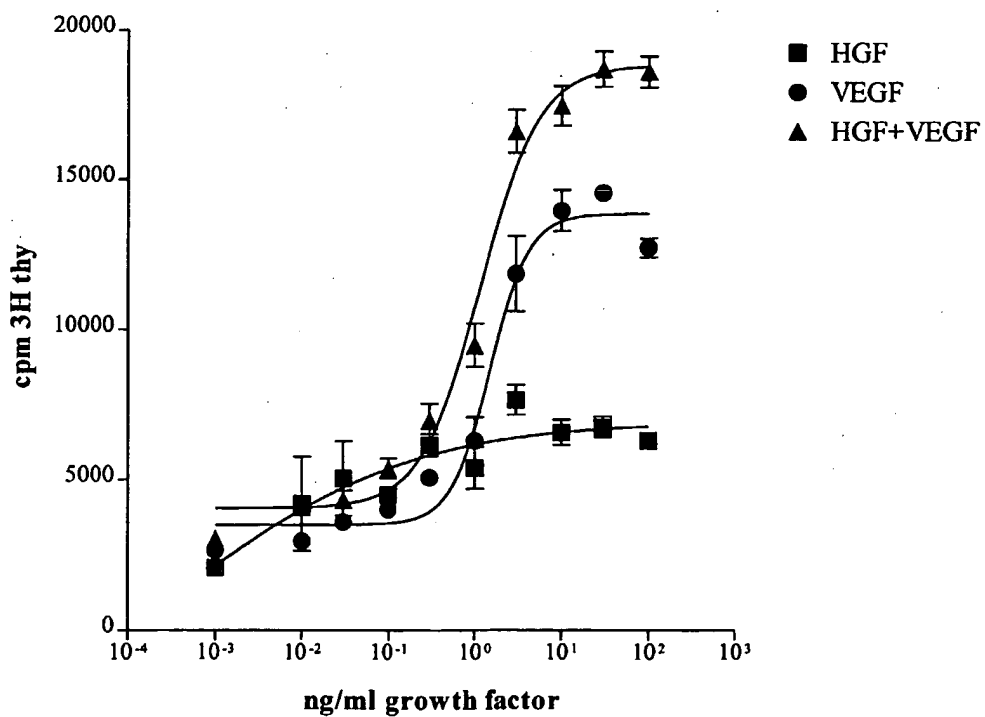


Fig. 13

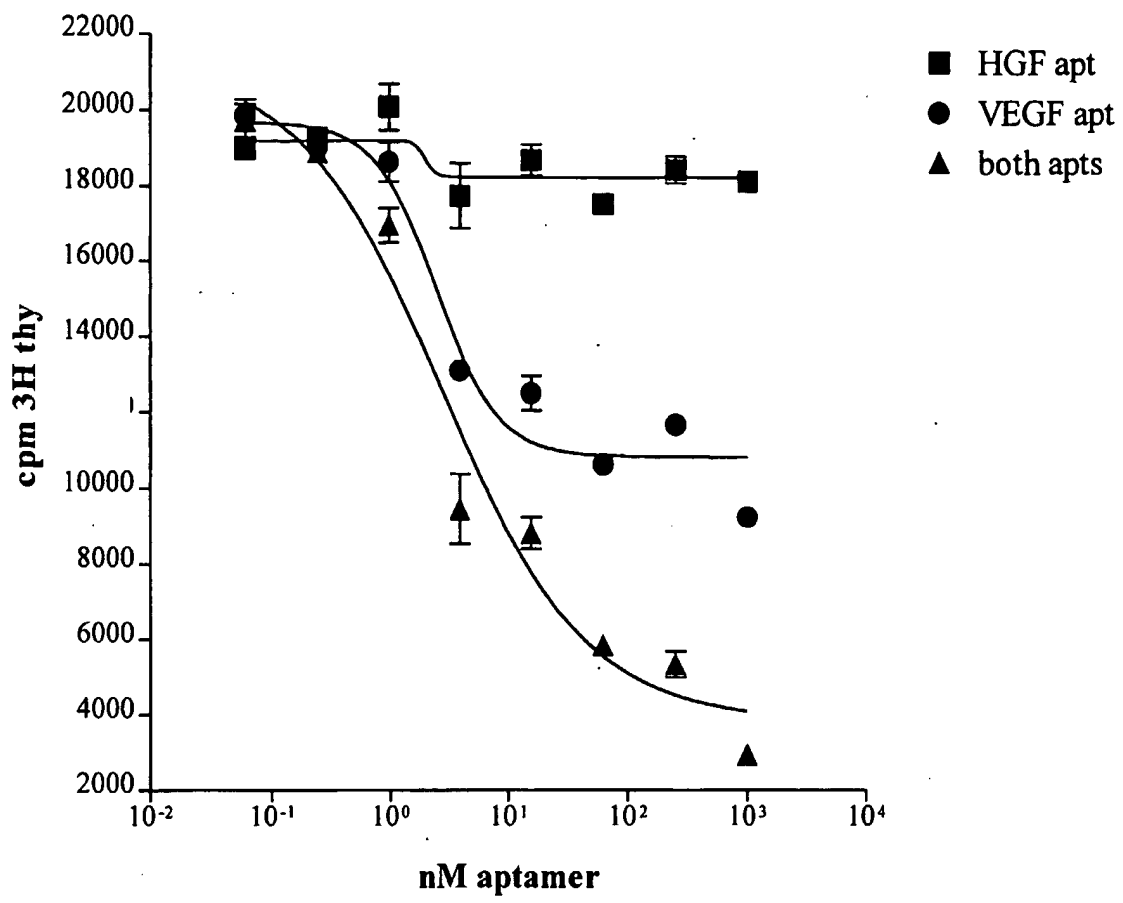


Fig. 14A

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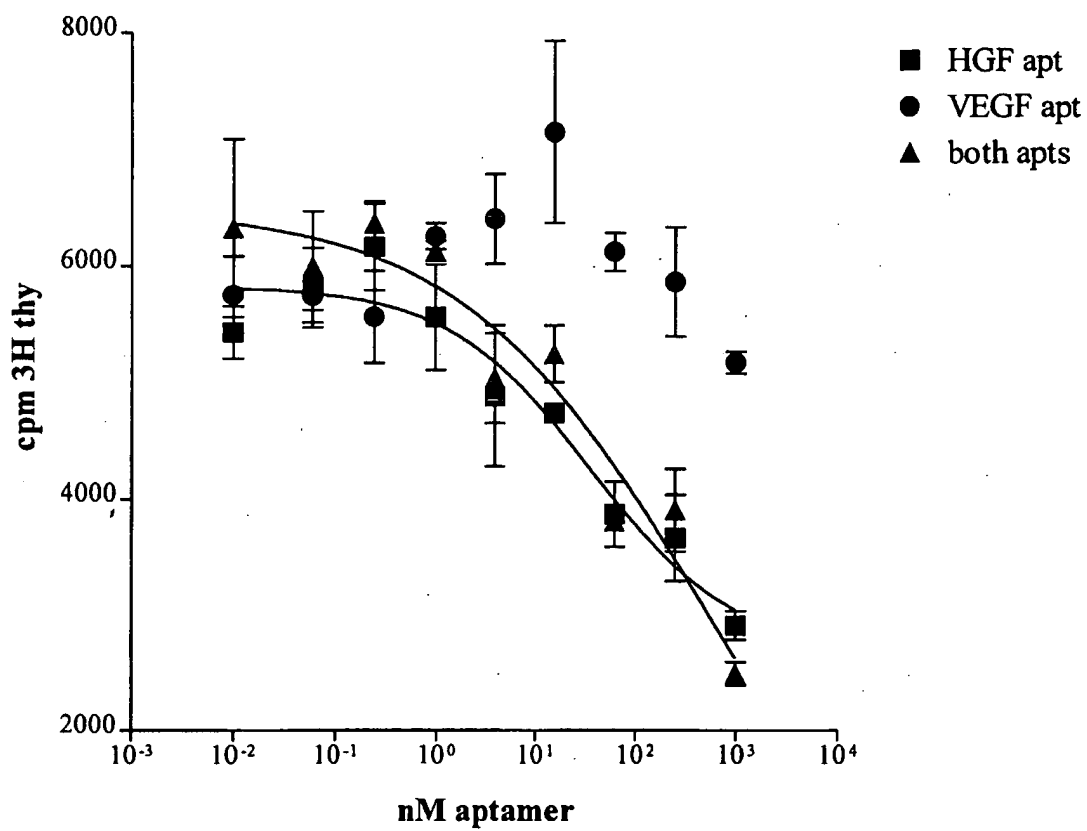


Fig. 14B

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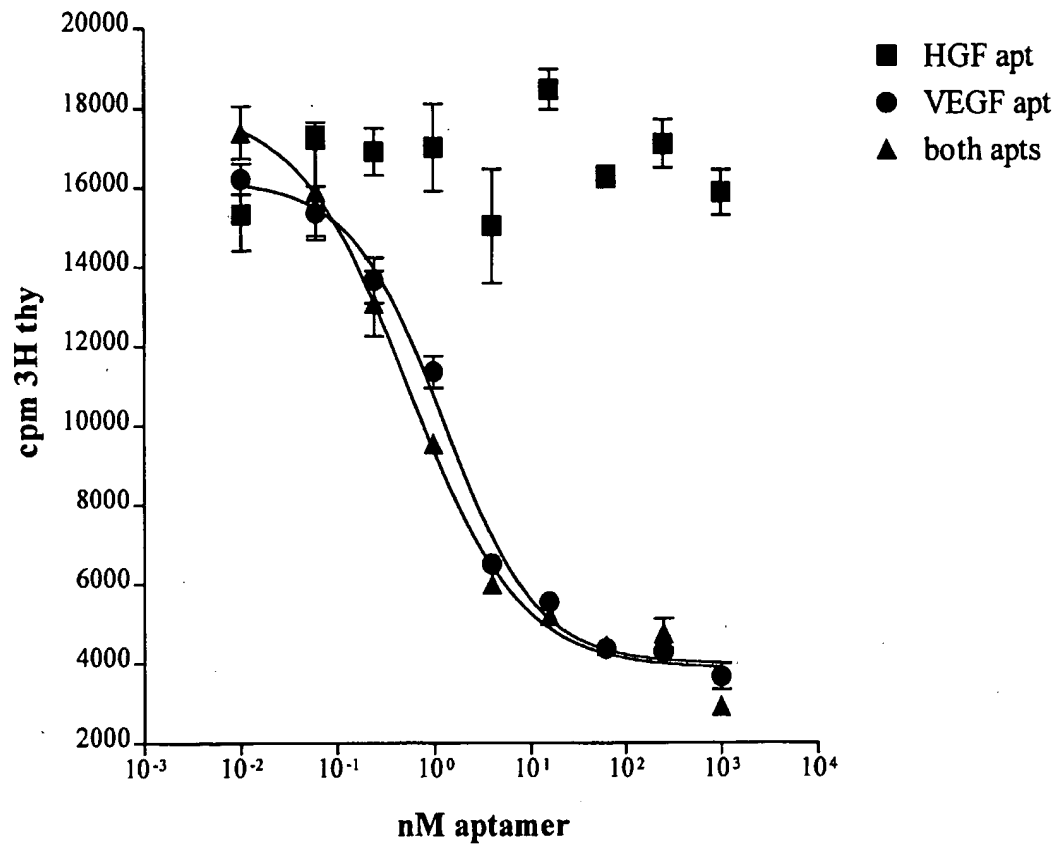


Fig. 14C



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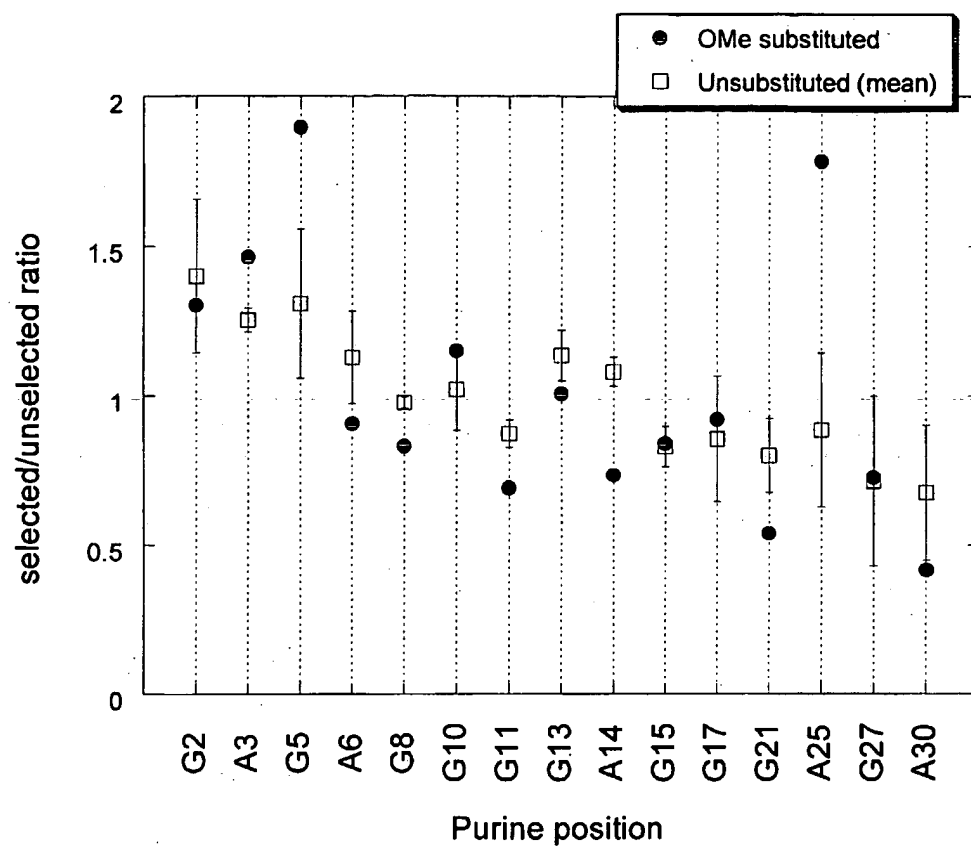


Fig. 15

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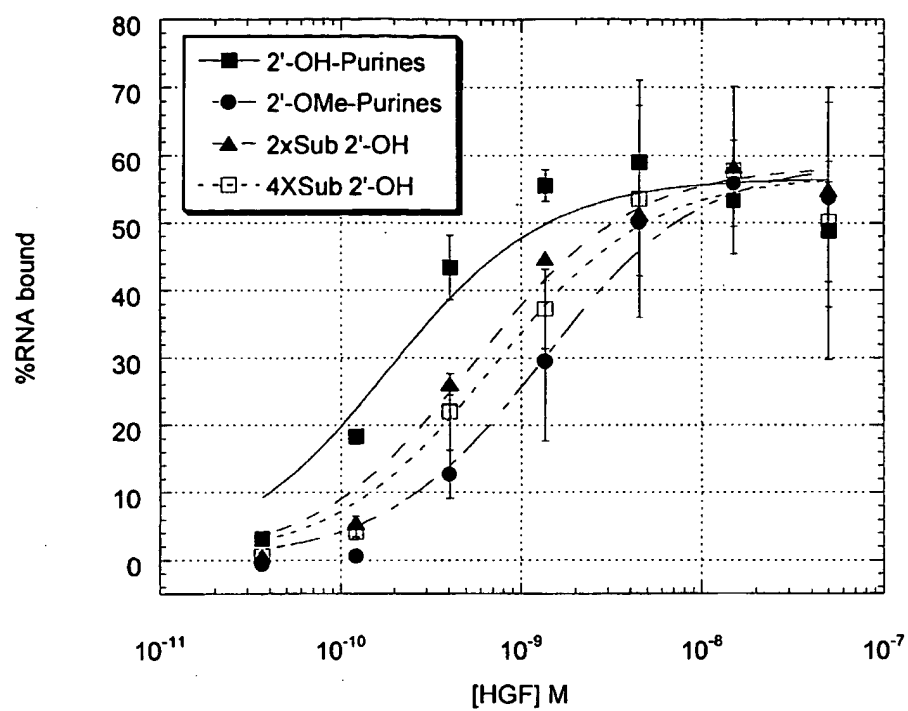


Fig. 16

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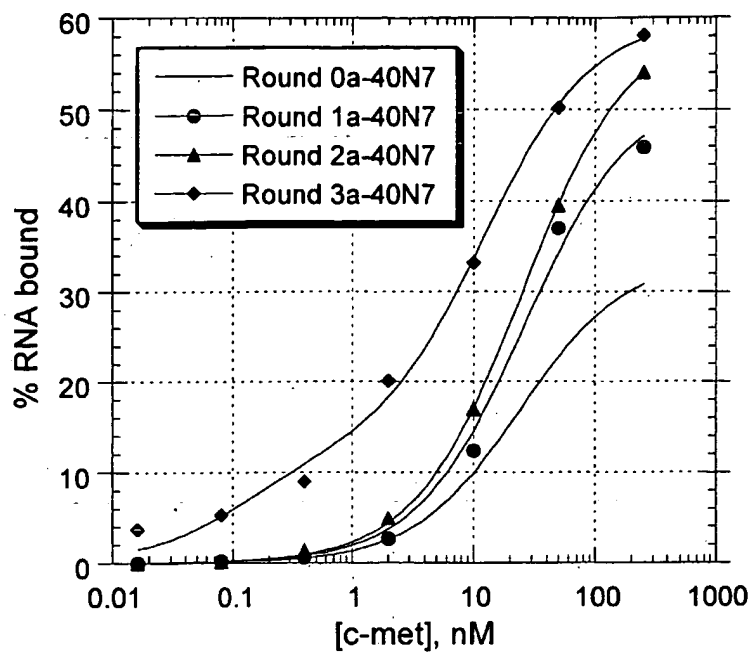


Fig. 17A

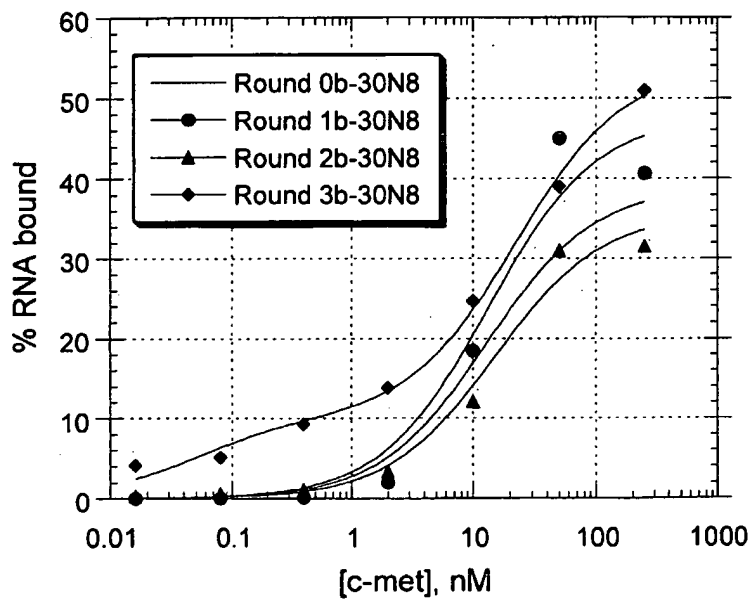


Fig. 17B

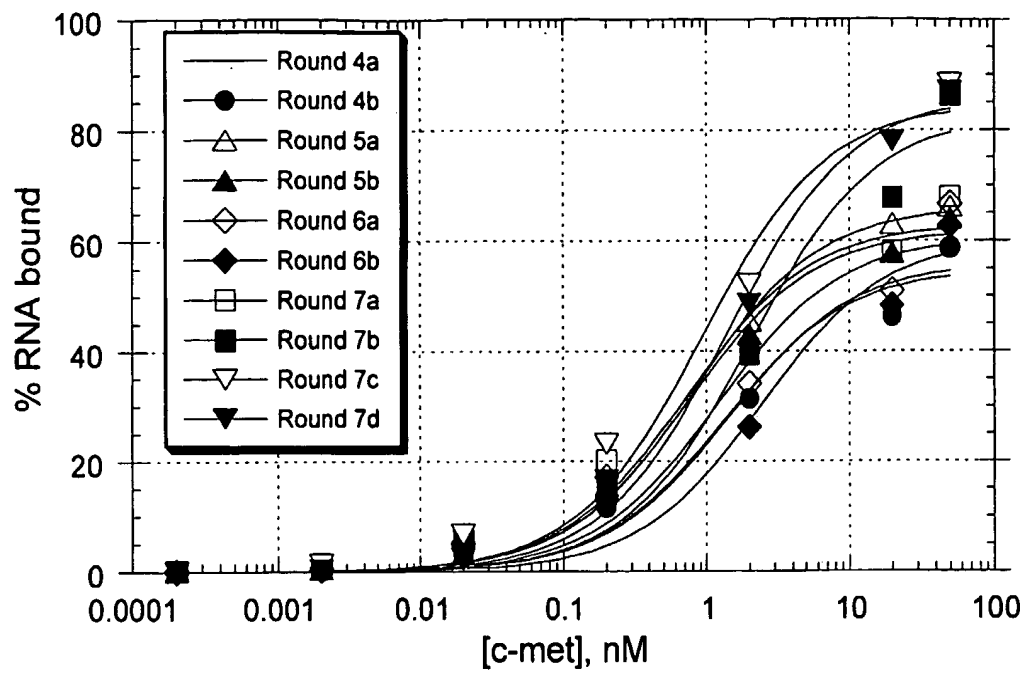


Fig. 17C

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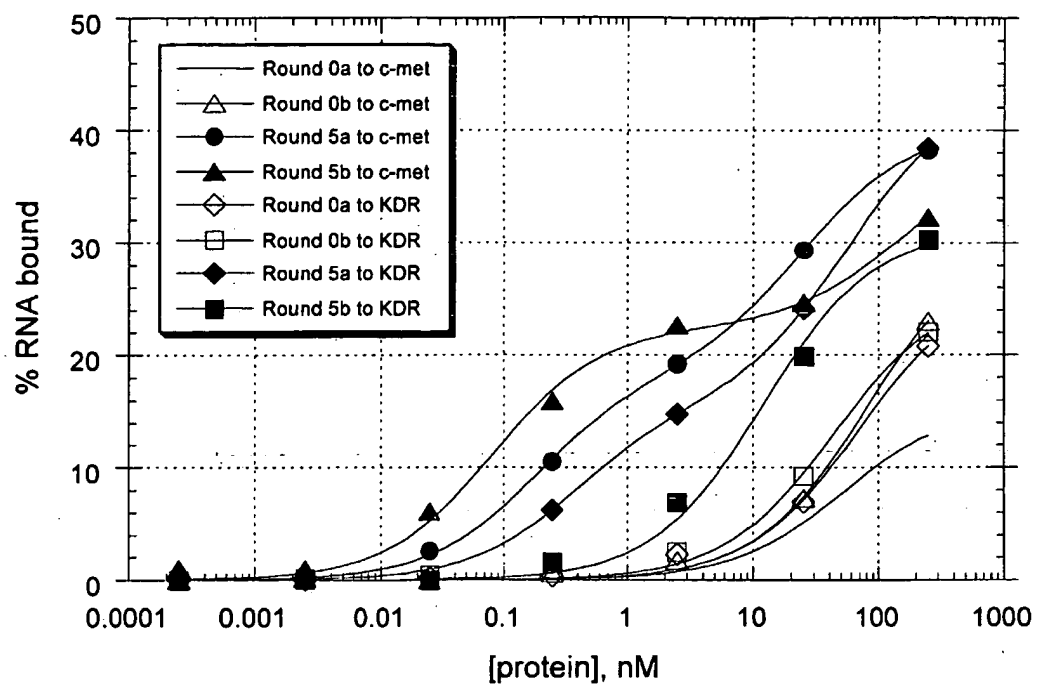


Fig. 18

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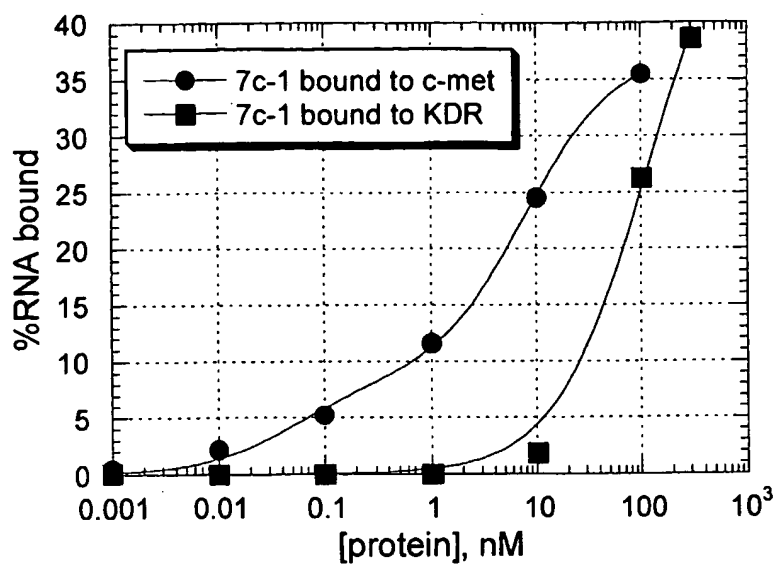


Fig. 19A

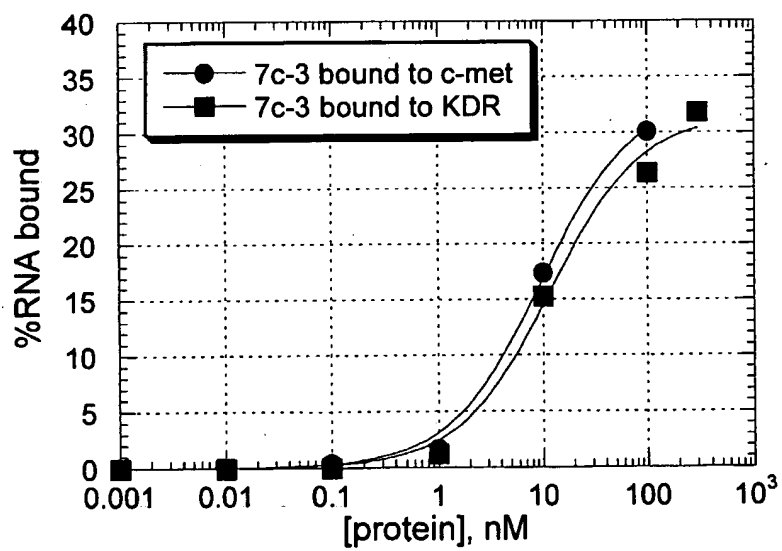


Fig. 19B

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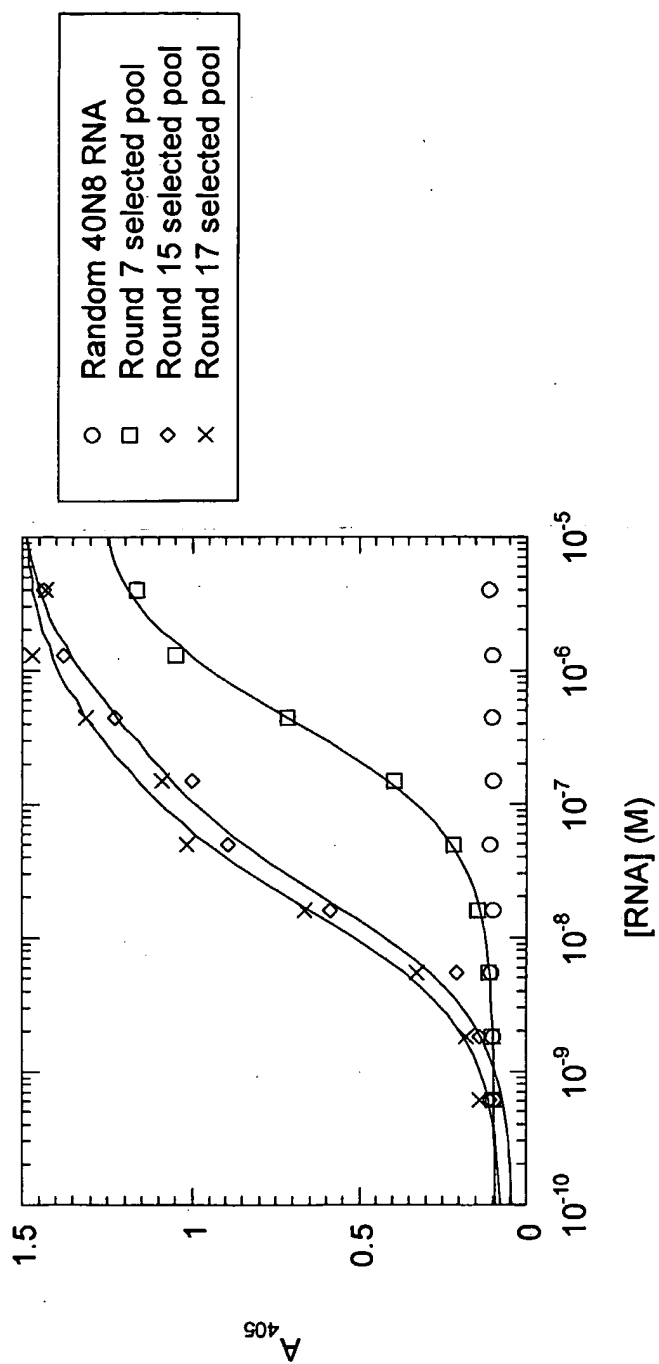


Fig. 20

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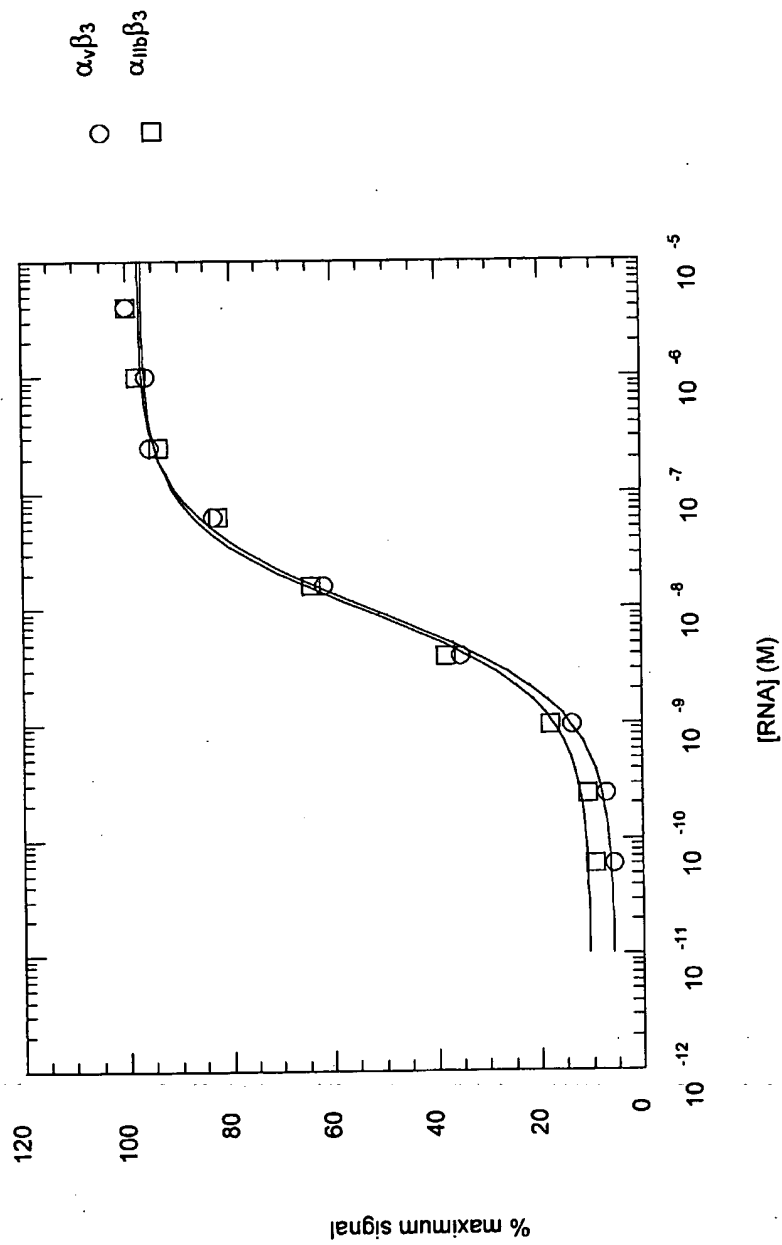


Fig. 21



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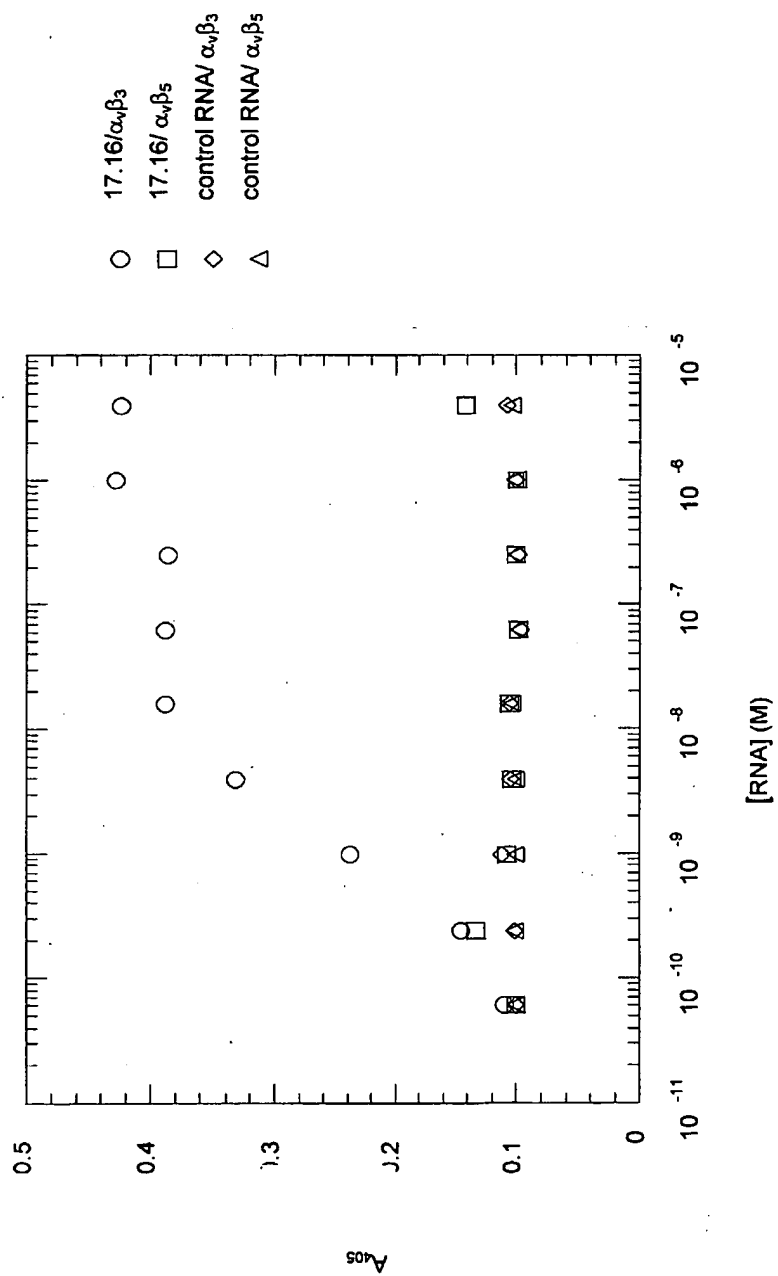


Fig. 22

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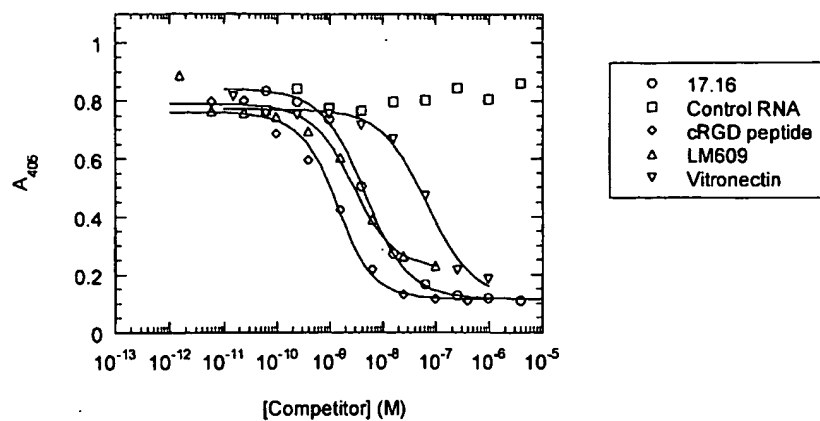


Fig. 23A

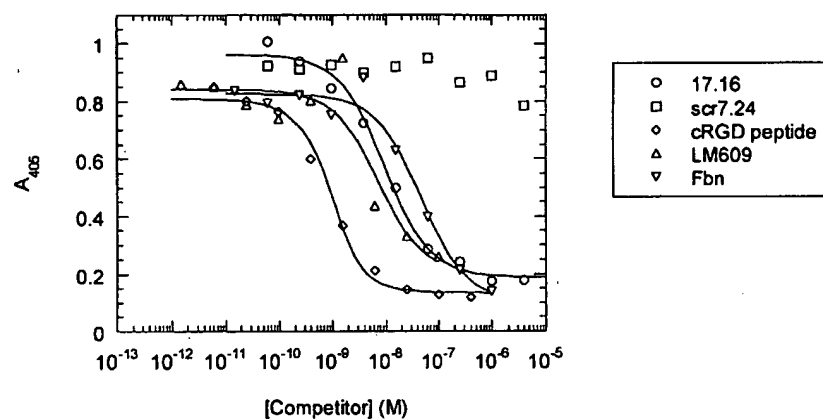


Fig. 23B

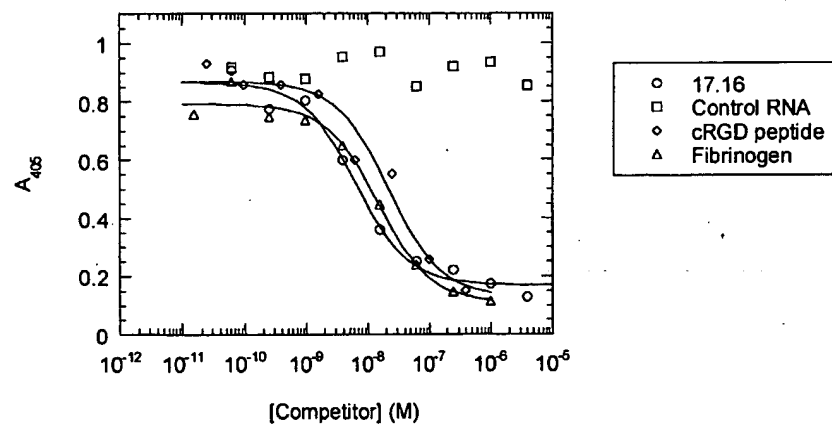


Fig. 23C

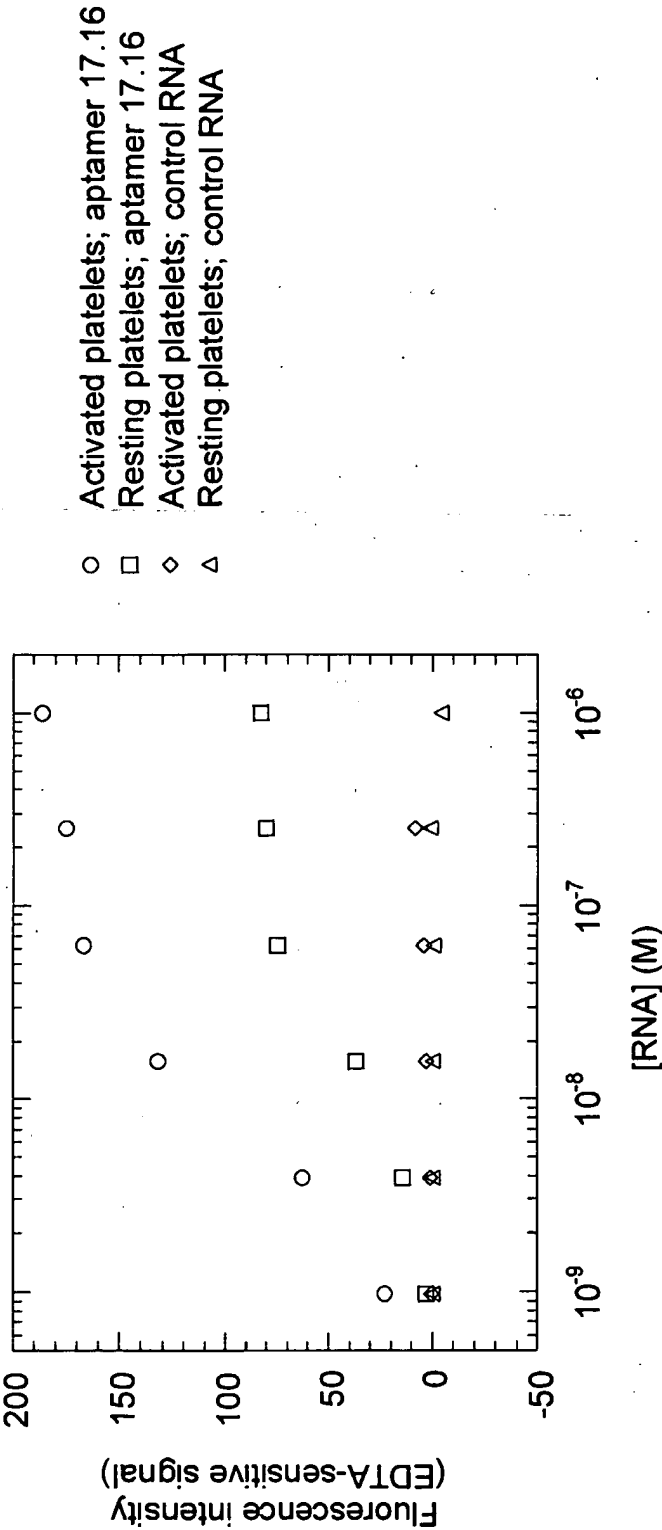


Fig. 24

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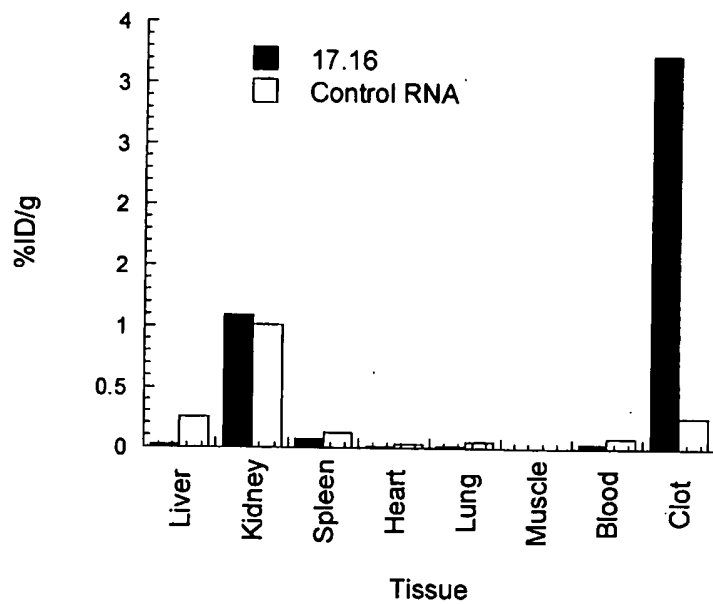


Fig. 25

## SEQUENCE LISTING

<110> NEXSTAR PHARMACEUTICALS, INC.

<120> Nucleic Acid Ligands Which Bind to Hepatocyte Growth Factor/Scatter Factor (HGF/SF) or its Receptor C-Met and to Integrins

<130> NEX 82/PCT

<140>

<141>

<150> 09/364,543

<151> 1999-07-29

<150> 09/364,539

<151> 1999-07-29

<160> 310

<170> PatentIn Ver. 2.0

<210> 1

<211> 78

<212> DNA

<213> Artificial Sequence

<220>

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<221> modified\_base

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nncagacgac ucgcccga 78

<210> 2

<211> 61

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic

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&lt;221&gt; modified\_base

&lt;222&gt; (1)..(61)

&lt;223&gt; All pyrimidines are 2'F.

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(61)

&lt;223&gt; N at positions 16-45 is any base.

&lt;400&gt; 2

gggaggacga ugcggnnnnn nnnnnnnnnn nnnnnnnnnn nnnnncagac gacucgcccg 60  
a 61

&lt;210&gt; 3

&lt;211&gt; 32

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

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&lt;210&gt; 4

&lt;211&gt; 16

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

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&lt;210&gt; 5

&lt;211&gt; 94

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(94)

<223> N at positions 41-70 is any base.

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nnnnnnnnnn ttcgacagga ggctcacaac aggc 94

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<211> 77

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

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<221> modified\_base

<222> (1)..(77)

<223> All pyrimidines are 2'F.

<220>

<221> modified\_base

<222> (1)..(77)

<223> N at positions 24-53 is any base.

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gggagauaag aaauaacgcu caannnnnnn nnnnnnnnnn nnnnnnnnnn nnnuucgaca 60  
ggaggcucac aacaggc 77

<210> 7

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<400> 7

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<210> 8

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<400> 8

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24

<210> 9

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

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<221> modified\_base

<222> (1)..(15)

<223> Purines and pyrimidines are 2'OMe; purines and pyrimidines at positions 1-11 are RNA; purines and pyrimidines at positions 12-15 are DNA.

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cccuccugcu acgcc

15

<210> 10

<211> 16

<212> DNA

<213> Artificial Sequence

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<221> modified\_base

<222> (1)..(16)

<223> Purines and pyrimidines are 2'OMe; purines and pyrimidines at positions 1-4 are DNA; purines and pyrimidines at positions 5-16 are RNA.

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16

<210> 11



<211> 34  
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Sequence

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<222> (1)..(34)  
<223> All pyrimidines are 2'F.

<220>  
<221> modified\_base  
<222> (1)..(34)  
<223> N at positions 3-32 is any base.

<400> 11  
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34

<210> 12  
<211> 61  
<212> RNA  
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Sequence

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<223> All pyrimidines are 2'F.

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a 61

<210> 13  
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<220>  
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Sequence

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<221> modified\_base

<222> (1)..(36)

<223> All pyrimidines are 2'F.

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36

<210> 14

<211> 28

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(28)

<223> All pyrimidines are 2'F.

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28

<210> 15

<211> 88

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(88)

<223> N at positions 33-72 is any base.

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nnnnnnnnnn nncagacgac ucgcccga 88

<210> 16

<211> 28

<212> RNA

<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(28)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 16

ggacgaugcg ggcggcucga ugucgucu

28

&lt;210&gt; 17

&lt;211&gt; 38

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(38)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 17

gggaggacga ugcgggcggc ucgaugucgu cuuauccc

38

&lt;210&gt; 18

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 18

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a

61

&lt;210&gt; 19

<211> 61  
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<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

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<222> (1)..(60)  
<223> All pyrimidines are 2'F.

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a 61

<210> 20  
<211> 61  
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<220>  
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Sequence

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<222> (1)..(60)  
<223> All pyrimidines are 2'F.

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a 61

<210> 21  
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<223> Description of Artificial Sequence: Synthetic  
Sequence

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<223> All pyrimidines are 2'F.

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a 61

<210> 22

<211> 61

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

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<222> (1)..(60)

<223> All pyrimidines are 2'F.

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a 61

<210> 23

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<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(60)

<223> All pyrimidines are 2'F.

<400> 23

gggaggacga ugcggcguga gccuauuuau gucaucaugu cuguccagac gacucgcccg 60  
a 61

<210> 24

<211> 61

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic

## Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 24

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a

61

&lt;210&gt; 25

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 25

gggaggacga ugcggcgaga gccuauuuau gucaucaugu cugugcagac gacucgcccg 60

a

61

&lt;210&gt; 26

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

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&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 26

gggaggacga ugcggcgaga gccuauuuau gucaucaugu cugugcagac gacucgcccg 60

a

61

&lt;210&gt; 27

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<220>  
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<221> modified\_base  
<222> (1)..(60)  
<223> All pyrimidines are 2'F.

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a 61

<210> 28  
<211> 61  
<212> RNA  
<213> Artificial Sequence

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Sequence

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<222> (1)..(60)  
<223> All pyrimidines are 2'F.

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a 61

<210> 29  
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<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
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<222> (1)..(60)  
<223> All pyrimidines are 2'F.

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a 61

<210> 30  
<211> 61  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(60)  
<223> All pyrimidines are 2'F.

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a 61

<210> 31  
<211> 61  
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Sequence

<220>  
<221> modified\_base  
<222> (1)..(60)  
<223> All pyrimidines are 2'F.

<400> 31  
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a 61

<210> 32  
<211> 61  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic



## Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 32

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a 61

&lt;210&gt; 33

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 33

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a 61

&lt;210&gt; 34

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
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&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 34

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a 61

&lt;210&gt; 35

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Sequence

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a 61

<210> 36  
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<220>  
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Sequence

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<210> 37  
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a 61

<210> 38

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<223> All pyrimidines are 2'F.

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a 61

<210> 39

<211> 61

<212> RNA

<213> Artificial Sequence

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Sequence

<220>

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<222> (1)..(60)

<223> All pyrimidines are 2'F.

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a 61

<210> 40

<211> 61

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<223> All pyrimidines are 2'F.

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a 61

<210> 41

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<223> All pyrimidines are 2'F.

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<210> 42

<211> 60

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<210> 43

<211> 59

<212> RNA

<213> Artificial Sequence

<220>

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<220>

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<210> 44

<211> 61

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<210> 45

<211> 61

<212> RNA

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a

61

&lt;210&gt; 46

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 46

gggaggacga ugcggcguga gccuaucau gucaucgugu gugcccagac gacucgcccg 60  
a 61

&lt;210&gt; 47

&lt;211&gt; 60

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(59)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 47

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&lt;210&gt; 48

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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Sequence

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a 61

<210> 49

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<212> RNA

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a 61

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<223> All pyrimidines are 2'F.

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a 61

<210> 51

<211> 57

<212> RNA

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&lt;220&gt;

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Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(56)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 51

gggaggacga ugcggcguga gccuauuuau gucaucgucu gcagacgacu cgcccga 57

&lt;210&gt; 52

&lt;211&gt; 60

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(59)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 52

gggaggacga ugcgguacagu augacuuaa uagcacguuc gcccagacg acucgcccga 60

&lt;210&gt; 53

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

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a

61

&lt;210&gt; 54



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a 61

<210> 56  
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&lt;400&gt; 56

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a 61

&lt;210&gt; 57

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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&lt;220&gt;

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&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 57

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a 61

&lt;210&gt; 58

&lt;211&gt; 62

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
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&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(61)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 58

gggaggacga ugcgggugac ucaaaauggu gaucucgau uccgccaga cgacucgccc 60  
ga 62

&lt;210&gt; 59

&lt;211&gt; 62

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
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<222> (1)..(61)

<223> All pyrimidines are 2'F.

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ga 62

<210> 60

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<212> RNA

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<223> All pyrimidines are 2'F.

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a 61

<210> 61

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<212> RNA

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<222> (1)..(60)

<223> All pyrimidines are 2'F.

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<210> 62

<211> 61

<212> RNA  
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<220>  
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<223> All pyrimidines are 2'F.

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a 61

<210> 63  
<211> 62  
<212> RNA  
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<222> (1)..(61)  
<223> All pyrimidines are 2'F.

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ga 62

<210> 64  
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<212> RNA  
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<220>  
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<210> 65  
<211> 61  
<212> RNA  
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<220>  
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a 61

<210> 66  
<211> 61  
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<220>  
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a 61

<210> 67  
<211> 61  
<212> RNA  
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<223> All pyrimidines are 2'F.

<400> 67

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<210> 68

<211> 61

<212> RNA

<213> Artificial Sequence

<220>

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<222> (1)..(60)

<223> All pyrimidines are 2'F.

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<210> 69

<211> 64

<212> RNA

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<222> (1)..(63)

<223> All pyrimindines are 2'F.

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<210> 70

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<210> 71  
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<210> 72  
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<223> All pyrimidines are 2'F.

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<210> 73  
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<223> All pyrimidines are 2'F.

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a 61

<210> 74  
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a 61

<210> 75  
<211> 59  
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<220>

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<210> 76

<211> 61

<212> RNA

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<220>

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<222> (1)..(60)

<223> All pyrimidines are 2'F.

<400> 76

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a 61

<210> 77

<211> 61

<212> RNA

<213> Artificial Sequence

<220>

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<223> All pyrimidines are 2'F.

<400> 77

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a 61

<210> 78

<211> 61

<212> RNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic  
Sequence

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<222> (1)..(60)

<223> All pyrimidines are 2'F.

<400> 78

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a 61

<210> 79

<211> 61

<212> RNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic  
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<220>

<221> modified\_base

<222> (1)..(60)

<223> All pyrimidines are 2'F.

<400> 79

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a 61

<210> 80

<211> 61

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(60)

<223> All pyrimidines are 2'F.

<400> 80

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a

61

&lt;210&gt; 81

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 81

gggaggacga ugcgggugga gucaaaucgu cuuguccuu guccucagac gacucgcccg 60  
a 61

&lt;210&gt; 82

&lt;211&gt; 61

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(60)

&lt;223&gt; All pyrimidines are 2'F.

&lt;400&gt; 82

gggaggacga ugcggguuug aguucugaca cgucuugucc caugccagac gacucgcccg 60  
a 61

&lt;210&gt; 83

&lt;211&gt; 64

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

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<210> 84  
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a 61

<210> 85  
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Sequence

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<400> 85  
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a 61

<210> 86  
<211> 61  
<212> RNA  
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<222> (1)..(60)

<223> All pyrimidines are 2'F.

<400> 86

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<210> 87

<211> 60

<212> RNA

<213> Artificial Sequence

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<222> (1)..(59)

<223> All pyrimidines are 2'F.

<400> 87

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<210> 88

<211> 61

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
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<220>

<221> modified\_base

<222> (1)..(60)

<223> All pyrimidines are 2'F.

<400> 88

gggaggacga ugcggaggac uaaucccuaa ggaauagcuu gccgcagac gacucgccc 60  
a 61

<210> 89  
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<210> 91  
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<210> 92

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<210> 93

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<210> 95

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&lt;400&gt; 111

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Sequence

<220>

<221> modified\_base

<222> (1)..(31)

<223> All pyrimidines are 2'F.

<400> 147

gcuggcucga ugucagguua uccuuugcc c

31

<210> 148

<211> 30

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(30)

<223> All pyrimidines are 2'F.

<400> 148

gugacucaaa auggugauc ucguuuccgc

30

<210> 149

<211> 30

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(30)

<223> All pyrimidines are 2'F.

<400> 149

ugaauuccuc uggcugaaaa ugacuuguc

30

<210> 150

<211> 30

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(30)

<223> All pyrimidines are 2'F.

<400> 150

guuugaguga cgaucgucuu gucccaugug

30

<210> 151

<211> 30

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<220>

<221> modified\_base

<222> (1)..(30)

<223> All pyrimidines are 2'F.

<400> 151

auugauucac ugcauccuug acucuucccc

30

<210> 152

<211> 28

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<220>

<221> modified\_base

<222> (1)..(28)

<223> All pyrimidines are 2'F.

<400> 152

cagacgacuc gcccgaaagga cgaugcgg

28

<210> 153

<211> 31

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<220>

<221> modified\_base

<222> (1)..(31)

<223> All pyrimidines are 2'F.

<400> 153

gaguuaauuu ucgucacccg uuccuuugcc c

31

<210> 154

<211> 29

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<220>

<221> modified\_base

<222> (1)..(29)

<223> All pyrimidines are 2'F.

<400> 154

acaguuuguc uucuaugcu cgucgcccc

29

<210> 155

<211> 30

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<220>

<221> modified\_base

<222> (1)..(30)

<223> All pyrimidines are 2'F.

<400> 155

ucagaaugac uuucuaugcu cgcuuucccc

30

<210> 156

<211> 37

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<220>

<221> modified\_base

<222> (1)..(37)

<223> All pyrimidines are 2'F. Bases at positions 1, 6, 13, 21, 33 are 2'OMe.

<400> 156

ggacgaugcg gcgagugccu guuuauauga ucguccg

37

<210> 157  
<211> 37  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(37)  
<223> All pyrimidines are 2'F. Bases at positions 2, 8,  
14, 25 are 2'OMe.

<400> 157  
ggacgaugcg gcgagugccu guuuauguca ucguccg . 37

<210> 158  
<211> 37  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(37)  
<223> All pyrimidines are 2'F. Bases at positions 3,  
10, 15 and 27 are 2'OMe.

<400> 158  
ggacgaugcg gcgagugccu guuuauguca ucguccg . 37

<210> 159  
<211> 37  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base

<222> (1)..(37)

<223> All pyrimidines are 2'F. Bases at positions 5,  
11, 17, 30 are 2'OMe.

<400> 159

ggacgaugcg gcgagugccu guuuauguca ucguccg

37

<210> 160

<211> 70

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(70)

<223> All pyrimidines are 2'F.

<400> 160

gggaggacga ugcgguuuga cuaugucuga cgggucugug gucaauuccg cccccagacg 60  
acucgcccga 70

<210> 161

<211> 71

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(71)

<223> All pyrimidines are 2'F.

<400> 161

gggaggacga ugcggauccg uguugauguc cauauaaccu uaucccgucg cccccagac 60  
gacucgcccg a 71

<210> 162

<211> 71

<212> RNA

<213> Artificial Sequence



<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<220>

<221> modified\_base

<222> (1)..(71)

<223> All pyrimidines are 2'F.

<400> 162

gggaggacga ugcggguguu gacuucuagc cagaauaaca uuuuguaccc cucccccagac 60  
gacucgcccga a 71

<210> 163

<211> 70

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<220>

<221> modified\_base

<222> (1)..(70)

<223> All pyrimidines are 2'F.

<400> 163

gggaggacga ugcgguuguu gagcuuuuga uagggcuugu ucuucgagcg uccccagacg 60  
acucgcccga 70

<210> 164

<211> 71

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Sequence

<220>

<221> modified\_base

<222> (1)..(71)

<223> All pyrimidines are 2'F.

<400> 164

gggaggacga ugcgguuauu uuggguuuuga ucguauuuac uucacccucc guccccagac 60  
gacucgcccga a 71

<210> 165  
<211> 71  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(71)  
<223> All pyrimidines are 2'F.

<400> 165  
gggaggacga ugcggcuccu uuuccgcuaa acaagaccac uuugagcccu gccccagac 60  
gacucgcccg a .71

<210> 166  
<211> 71  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(71)  
<223> All pyrimidines are 2'F.

<400> 166  
gggaggacga ugcggccacc ucguuacgua cugauuuugg caucgcaguu ugccccagac 60  
gacucgcccg a 71

<210> 167  
<211> 73  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base

<222> (1)..(73)

<223> All pyrimidines are 2'F.

<400> 167

gggaggacga ugcgggggca ccucgauacg uacugauuuu gaauaucagu uagccccag 60  
acgacugcc cga 73

<210> 168

<211> 71

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(71)

<223> All pyrimidines are 2'F.

<400> 168

gggaggacga ugcggcgauu cgucguauag aaugauuug aaugcaccuc cuccccagac 60  
gacucgccc a 71

<210> 169

<211> 71

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(71)

<223> All pyrimidines are 2'F.

<400> 169

gggaggacga ugcggugugu uuguguguug uguuuguuau uccuguuugu guccucagac 60  
gacucgccc a 71

<210> 170

<211> 72

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(72)

<223> All pyrimidines are 2'F.

<400> 170

gggaggacga ugcgguccggu cguaaaaaaau cguugguguc uaucuauugu ucuccccaga 60  
cgacucgccc ga 72

<210> 171

<211> 70

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(70)

<223> All pyrimidines are 2'F.

<400> 171

gggaggacga ugcgguccguc cagaggaacc aucguuuacu ucauuuauuc gccccagacg 60  
acucgcccga 70

<210> 172

<211> 72

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(72)

<223> All pyrimidines are 2'F.

<400> 172

gggaggacga ugcgguccguc cuuaggaacc aucgucuaua ucccauucug acugcccaga 60  
cgacucgccc ga 72

<210> 173  
<211> 71  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(71)  
<223> All pyrimidines are 2'F.

<400> 173  
gggaggacga ugcggugcuc cucaggaacc aucguuuuuc ccauguccuu cugcccagac 60  
gacucgcccg a 71

<210> 174  
<211> 71  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(71)  
<223> All pyrimidines are 2'F.

<400> 174  
gggaggacga ugcggugcuc cuuggauuac caaggaacca uuuuccucua cccccagac 60  
gacucgcccg a 71

<210> 175  
<211> 76  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base

<222> (1)..(76)

<223> All pyrimidines are 2'F.

<400> 175

gggagauaag aaauaacgcu caagugcuca uuacgaacu gaccgaugcc uauucgacag 60  
gaggcucaca acaggc 76

<210> 176

<211> 77

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(77)

<223> All pyrimidines are 2'F.

<400> 176

gggagauaag aaauaacgcu caaggugcuc auuacgaacu ugaccgaagc cuauucgaca 60  
ggaggcucac aacaggc 77

<210> 177

<211> 77

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(77)

<223> All pyrimidines are 2'F.

<400> 177

gggagauaag aaauaacgcu caaggugcuc auuacgaacu ugaccgaugc cuauucgaca 60  
ggaggcucac aacaggc 77

<210> 178

<211> 74

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(74)

<223> All pyrimidines are 2'F.

<400> 178

gggagauaag aaauaacgcu caaagugcuc caaugaacuu ugcucgcuga uucgacagga 60  
ggcucacaac aggc 74

<210> 179

<211> 77

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(77)

<223> All pyrimidines are 2'F.

<400> 179

gggagauaag aaauaacgcu caaggugcuc cguuugaac ugaucggua ggauucgaca 60  
ggaggcucac aacaggc 77

<210> 180

<211> 76

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(76)

<223> All pyrimidines are 2'F.

<400> 180

gggagauaag aaauaacgcu caagugcuca uucagaacuu gacguauaac cauucgacag 60  
gaggcucaca acaggc 76

<210> 181  
<211> 77  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(77)  
<223> All pyrimidines are 2'F.

<400> 181  
gggagauaag aaauaacgcu caaggugcuc cuuaggaacu ugaccguccg ccauucgaca 60  
ggaggcucac aacaggc 77

<210> 182  
<211> 77  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(77)  
<223> All pyrimidines are 2'F.

<400> 182  
gggagauaag aaauaacgcu caaggugcuc uccacuaacc aaguggaacc uuguucgaca 60  
ggaggcucac aacaggc 77

<210> 183  
<211> 66  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base



<222> (1)..(66)

<223> All pyrimidines are 2'F.

<400> 183

gggagauaag aaauaacgcu caagugcucu ugaacuugac cguucgacag gaggcucaca 60  
acaggc 66

<210> 184

<211> 77

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(77)

<223> All pyrimidines are 2'F.

<400> 184

gggagauaag aaauaacgcu caaacgauaa gugggaguga guaaguuuga guauucgaca 60  
ggaggcucac aacaggc 77

<210> 185

<211> 77

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(77)

<223> All pyrimidines are 2'F.

<400> 185

gggagauaag aaauaacgcu caaccuagac cccagguuc cucccccacua gucuucgaca 60  
ggaggcucac aacaggc 77

<210> 186

<211> 36

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(36)

<223> All pyrimidines are 2'F. Purines at positions 1,  
2, 6, 8, 11, 13-15, 17, 21, 27, 30, 33 are 2'OMe.

<400> 186

ggacgaugcg gcgagugccu guuuauguca ucgucc

36

<210> 187

<211> 36

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(36)

<223> All pyrimidines are 2'F. Purines at 1-3, 6, 8,  
10-11, 13-15, 17, 21, 27, 30, 33 are 2'OMe.

<400> 187

ggacgaugcg gcgagugccu guuuauguca ucgucc

36

<210> 188

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<400> 188

acgagtttat cgaaaaagaa cgatggttcc aatggagca

39

<210> 189

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<400> 189

cgcaggatcc taatacgact cactata

27

<210> 190

<211> 104

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(104)

<223> N at positions 41-80 is any base

<400> 190

ttatacgact cactataggg agacaagaat aaacgctcaa nnnnnnnnnn nnnnnnnnnn 60  
nnnnnnnnnn nnnnnnnnnn ttcgacagga ggctcacaac aggc 104

<210> 191

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<400> 191

gcctgttgtg agcctcctgt cgaa

24

<210> 192

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<400> 192

taatacgact cactataggg agacaagaat aaacgctcaa

40

<210> 193  
<211> 87  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(87)  
<223> All pyrimidines are 2' F.

<400> 193  
gggagacaag aaauaagcgc caacguugaa ugcugcauua uggaguauug accgcuacau 60  
cccuucgaca ggaggcucac aacaggc 87

<210> 194  
<211> 85  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(85)  
<223> All pyrimidines are 2' F.

<400> 194  
gggagacaag aaauaacgcgc caauucucagc uuguuuuagg gcuuauauga gcgcuuuaua 60  
cccuucgaca ggaggcucaa caggc 85

<210> 195  
<211> 85  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base

<222> (1)..(85)

<223> All pyrimidines are 2' F.

<400> 195

gggagacaag aaauaacgcu caauucaacg cuguuuaagg gcuuauauga gcgcguuaua 60  
ccuucgaca ggaggcucaa caggc 85

<210> 196

<211> 87

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(87)

<223> All pyrimidines are 2' F.

<400> 196

gggagacaag aaauaacgcu caauucaacg cuguuuaagg gcuuauauga gcgcguuaca 60  
ccuucgaca ggaggcucac aacaggc 87

<210> 197

<211> 85

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(85)

<223> All pyrimidines are 2' F.

<400> 197

gggagacaag aaauaacgcu caauucaacg cuguucaagg gcuuauauga gcgcguuaua 60  
ccuucgaca ggaggcucaa caggc 85

<210> 198

<211> 87

<212> RNA

<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(87)

&lt;223&gt; All pyrimidines are 2' F.

&lt;400&gt; 198

gggagacaag aaauaacgcu caauucaacg auguuuaagg gcuuauauga gcgcguuaua 60  
cccuucgaca ggaggcucac aacaggc 87

&lt;210&gt; 199

&lt;211&gt; 85

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(85)

&lt;223&gt; All pyrimidines are 2' F.

&lt;400&gt; 199

gggagacaag aaauaacgcu caauucauga aguccaagg cuuauaugag gcgcguuauac 60  
ccuugacagg aggcucacaa caggc 85

&lt;210&gt; 200

&lt;211&gt; 86

&lt;212&gt; RNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Sequence

&lt;220&gt;

&lt;221&gt; modified\_base

&lt;222&gt; (1)..(86)

&lt;223&gt; All pyrimidines are 2' F.

&lt;400&gt; 200

gggagacaag aaauaacgcu caauucaacg cugucaaagg gcuuauauga gcgcguuaua 60  
cccuugacag gaggcucaca acaggc 86

<210> 201  
<211> 86  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(86)  
<223> All pyrimidines are 2' F.

<400> 201  
gggagacaag aaauaacgcu caauuaacgu uguucaaggg uuuaauaugag ugcguuauac 60  
ccuucgacag gaggcucaca acaggc 86

<210> 202  
<211> 87  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base  
<222> (1)..(87)  
<223> All pyrimidines are 2' F.

<400> 202  
gggagacaag aaauaacgcu caauucaacg cuguccaagg gcuaauauga gcgcguuaua 60  
ccuucgaca ggaggcucac aacaggc 87

<210> 203  
<211> 87  
<212> RNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>  
<221> modified\_base

<222> (1)..(87)

<223> All pyrimidines are 2' F.

<400> 203

gggagacaag aaauaacgcu caauucaacg gauguccaag ggcuuuauga gcgcguuaua 60  
cccuucgaca ggaggcucac aacaggc 87

<210> 204

<211> 87

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(87)

<223> All pyrimidines are 2' F.

<400> 204

gggagacaag aaauaacgcu caauucgacg cuguucaagg gcuuauauga gcgcguuaua 60  
uccuucgaca ggaggcucac aacaggc 87

<210> 205

<211> 87

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(87)

<223> All pyrimidines are 2' F.

<400> 205

gggagacaag aaauaacgcu caauucgacg cuguucaagg gcuuauauga gcgcguuaua 60  
cccuucgaca ggaggcucac aacaggc 87

<210> 206

<211> 87

<212> RNA

<213> Artificial Sequence



<220>

<223> Description of Artificial Sequence: Synthetic  
Sequence

<220>

<221> modified\_base

<222> (1)..(87)

<223> All pyrimidines are 2' F.

<400> 206

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cccuucgaca ggaggcucac aacaggc 87

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&lt;400&gt; 231

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/20139

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : C07H 21/04, 21/02; A61K 48/00; C12Q 1/68; C12N 15/85, 15/86  
US CL : 435/6, 325, 375; 536/23.1, 24.5, 24.3, 24.31, 24.33; 514, 44  
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 325, 375; 536/23.1, 24.5, 24.3, 24.31, 24.33; 514, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
STN, BIOSIS, MEDLINE, LIFESCI, EMBASE, USPATFULL

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DAVIS ET AL. Identifying Concensus Patterns and Secondary Structure in SELEX Sequence Sets. Methods in Enzymology. 1996, Vol. 267, pages 302-314, see especially pages 312-314.	40-47
Y,P	TUCKER ET AL. Detection and plasma pharmacokinetics of an anti-vascular endothelial growth factor oligonucleotide-aptamer (NX1838) in rhesus monkeys. Journal of Chromatography. 10 September 1999, Vol. 732, No. 1, pages 203-212, see entire document.	46-47
Y	GREEN ET AL. Nuclease-resistant nucleic acid ligands to vascular permeability facotr/vascular endothelial growth factor. Chemistry and Biology. October 1995, Vol. 2, No. 10, pages 683-695, see entire document.	40-47

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

•	Special categories of cited documents:	•T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

27 AUGUST 2000

Date of mailing of the international search report

13 OCT 2000

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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FLOEGE ET AL. Novel Approach to Specific Growth Factor Inhibition in Vivo. American Journal of Pathology. 01 January 1999, Vol. 154, No. 1, pages 169-179, see entire document.	40-47
Y	WO 91/19813 A1 (THE UNIVERSITY OF COLORADO FOUNDATION, INC.) 26 December 1991 (26-12-91), see entire document.	1-17, 20-34, 48-66